

**Nanoformulation of cartap hydrochloride and its effect on growth and
physiology of *Anabaena variabilis* ARM 441**

A thesis submitted in fulfillment of the
requirements for the award of the degree of

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**



**THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY**
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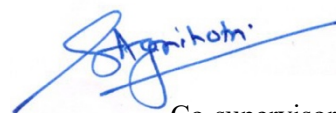
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CERTIFICATE

Certified that the thesis entitled “Nanoformulation of cartap hydrochloride and its effect on growth and physiology of *Anabaena variabilis* ARM441” submitted by Ms. Ishtpreet Kaur, in fulfilment of the requirement for the award of the degree of Doctor of Philosophy in Biotechnology, Thapar Institute of Engineering & Technology (Deemed to be University), Patiala, Punjab, India is a record of the candidate's own independent and original research work carried out by her under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.



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DECLARATION

I hereby declare that the work presented in this thesis entitled “Nanoformulation of cartap hydrochloride and its effect on growth and physiology of *Anabaena variabilis* ARM441” submitted by me for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology (Deemed to be University), Patiala, Punjab and Dr. Shekhar Agnihotri, Assistant professor, Department of Agriculture & Environmental Sciences, National Institute of Food Technology Entrepreneurship and Management, Kundli, Sonapat Haryana. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or Abroad.



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ACKNOWLEDGEMENT

The time has eventually arrived when my journey of PhD has reached the other end of the stupendous road of research. Being the most remarkable stretch traversed so far, not just gave me the academic knowledge, rather taught me different aspects of life by helping me to grow in stature, building invaluable self-confidence, as well as vision and determination to achieve substantially big in life.

I would like to sincerely express my gratitude and respect to my supervisor **Dr. Dinesh Goyal** for his keen interest and valuable guidance, strong motivation and constant encouragement throughout my journey in TIET, Patiala. His continuous support, great patience, immense knowledge, constructive criticism and myriad useful suggestions helped me a lot making my PhD easier and comfortable. His contribution and faith in me, helped me to smoothly sail through this journey. I greatly appreciate the freedom he gave me to pursue my independent work and find my own path during this research endeavour.

I also wish to express thanks to my co-supervisor **Dr. Shekhar Agnihotri** for his guidance valuable suggestions and comments from time to time.

I am highly grateful to the Director, **Thapar Institute of Engineering & Technology, Patiala**, for providing infrastructure, financial support and helping me towards the smooth and timely completion of my research work. I would like to thank Science & Technology Entrepreneurs Park (**STEP**), TIET for providing me necessary equipment and facilities to carry out this work. I extend my sincere words of thanks to Dean (Research and Sponsored Projects) for his support towards the completion of my work.

I wish to express my deep sense of humbleness to **Dr. M.S Reddy**, Head of the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala (Punjab) for his support. I extend my thanks to all the faculty members of Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala for providing necessary guidance during my research work.

My special word of thanks should also go to members of my doctoral committee **Dr. N. Das**, **Dr. Priyankar Dey**, DBT, TIET and **Dr. S.D. Tiwari**, School of Physics and Material

Science, for their invaluable suggestions, constant support and encouragement during the course of my study.

Words cannot express how indebted I am to **Simarpreet**, my bestie, who helping and their ungrudging assistance. This magnificent period would have been incomplete without **Nirmalya Halder and Harmeet**, TIET colleagues. I express my esteem and profound sense of gratitude to Ms. **Prerna** for her able guidance. I owe a word of thanks to my lab mates, **Jyotika, Ravneet and Purnima** for their generous help and support in infinite number of ways every time and anytime. I feel lacunae of words to express my most heartfelt and cordial thanks to all my **friends Devender and Parmandeep** who has always been a source of inspiration for me, stood by my side at the toughest times

I am very thankful to **Mrs. Manjula Kamra** for timely help and cooperation. Most of the work would have been incomplete without the sincere support of Lab assistants. So, I owe a word of thanks to **Mr. Ram Newal (Babban ji)** and all the **non-teaching staff** for helping and their ungrudging assistance.

I find it difficult to pen down my deepest sense of indebtedness towards my beloved parents and in-laws who soulfully provided me their constant support and right impetus to undertake the challenge of this proportion like all other spheres of life, for that I cannot measure but treasure. I remember their constant support when I encountered difficulties during my research. Their encouragement never made me feel alone and only supported me to act high. They believed in me before I believed in myself. I highly appreciate unsolicited cooperation and cheery assistance of my husband **Jagjot Singh**. I wish to extend a very special thanks to my husband for his unrelenting support, optimism and giving me immense time to complete this research work. I would also like to thank to my daughter **Pavit**, for giving me unlimited happiness and pleasure when I felt low and my enthusiasm waned. I consider myself luckiest in the world for the everlasting love and support of my entire family standing behind me. Words fail to express my deepest gratitude to my sister **Harmanpreet Kaur** for her all-time emotional support, constant care and love.

I wish to acknowledge all those, whose names have not been figured here, but who helped me in any form during the period of my research work.

Lastly, I am thankful to God, the Almighty, for showering countless blessings and granting me good health, knowledge, strength, and patience to work through all these years, so that today I can stand proud with my head held high.

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ABSTRACT

Insecticides are extensively applied in crop production to combat crop loss due to pest infestation and sometimes at higher concentration than their recommended doses, which has become an environmental hazard. Sustainable agricultural practices highlight minimal application of insecticides at low concentration. Use of controlled release formulations (CRFs) of pesticides in which active compound is associated with inert materials has emerged as an appealing alternative. Cartap hydrochloride is a moderately hazardous nereistoxin analogue insecticide that is predominantly applied in paddy fields of India, at a recommended dose of $10\text{ }\mu\text{g ml}^{-1}$ to kill chewing and sucking insect pests of rice crop. Nanoformulation of cartap hydrochloride using two natural polymers chitosan and alginate was done and study its release kinetics under *in vitro* conditions. Prior to that overall impact of cartap hydrochloride induced stress response and physiology of *Anabaena variabilis* ARM 441 were studied under pure conditions, a filamentous heterocystous cyanobacterium commonly used as algal biofertilizer in paddy cultivation.

Anabaena sp. could tolerate commercial grade insecticide up to $30\text{ }\mu\text{g ml}^{-1}$. However, at the recommended dose of $10\text{ }\mu\text{g ml}^{-1}$, it caused reduction in algal growth, total nitrogen and heterocyst frequency by 47.28, 24.29 and 17.72% respectively, as well as photosynthetic pigments under pure culture conditions. Increased concentrations of cartap resulted in a decrease in total protein content while increasing carbohydrate content. Scanning electron micrographs revealed cell rupture and breakage in filaments due to cartap exposure with the formation of akinetes. Cartap hydrochloride induced stress, since levels of superoxide dismutase, peroxidase and catalase were increased by 108.57, 187.5 and 117% respectively. Generation of superoxide radicals and hydrogen peroxide were also increased by 152.48 and 34% respectively. Lipid peroxidation was increased by 31.03%, whereas there was decline in ascorbate content by 48.45%, however the glutathione content was increased by 128.57%. The effect of cartap was strongest on NiR activity, followed by GS activity, and NR activity, among these three enzymes. To check whether the cyanobacterium was resistant to cartap or utilized it intracellularly, cyanobacterium was grown in medium supplemented with cartap ranging from 5, 10, $30\text{ }\mu\text{g ml}^{-1}$. The cyanobacterium removed cartap by 67, 55.8, and 50.43% in 5, 10, $30\text{ }\mu\text{g ml}^{-1}$ after 24 hours. Increase in osmolytes such as proline from 8.6 to 32.8% and sucrose from 61.22 to 90.13% indicates their possible role in overcoming cartap induced oxidative stress and can be helpful in assessing its detrimental effect on *Anabaena variabilis*

ARM 441, since cyanobacterial biofertilizers are purposely used in paddy fields as nitrogen contributors.

In order to reduce toxicity of cartap and for its controlled delivery, two types of insecticidal nanoformulations were developed using chitosan tripolyphosphate and chitosan alginate with help of ionic gelation and polyelectrolyte complexation method. These nanoformulations were characterized by dynamic light scattering (DLS), field emission scanning electron microscope (FESEM), X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectra. The FESEM images revealed that the size of chitosan tripolyphosphate (CS-TPP) nanospheres (nps) was in range of 117.01-185.27 nm, whereas cartap hydrochloride entrapped chitosan tripolyphosphate (C-CS-TPP) nanospheres had a size of 163.50-276.74 nm. However, the size of chitosan alginate (CS-ALG) nanospheres (nps) was in range of 101.66 -158.33 nm, whereas cartap hydrochloride entrapped chitosan alginate (C-CS-ALG) nanospheres had a size of 107.58 -173.07 nm as revealed by FESEM. FTIR results confirmed loading of cartap hydrochloride into both type of nanospheres. The nanospheres showed encapsulation efficiency of 86.1% and 76.19% in C-CS-TPP and C-CS-ALG and were stable for 30 days at ambient temperature. *In-vitro* release kinetics of insecticide from nanospheres followed a non Fickian diffusion mechanism in C-CS-TPP but super case II transport mechanism was followed by C-CS-ALG nanospheres.

With the application of such control release nanoformulations, it is possible to reduce the frequency of field application of insecticide due to its slow release to the target organism, which is economical as well as environmentally safe. Encapsulating cartap hydrochloride in a single carrier system can have prolong activity against targeted organisms at low doses of insecticide, and will also allow to proliferate cyanobacteria in paddy fields because of slow release of insecticide without negatively affecting the cyanobacterial flora. These nanoformulations because of their biodegradability, biocompatibility, greater stability, low toxicity and simple synthesis process, offers valuable tool for pesticide delivery systems.

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LIST OF ABBREVIATIONS

ALG	Alginate
APC	Allophycocyanin
BHC	Benzene hexachloride
C	Cartap hydrochloride
CaCl ₂	Calcium chloride
CAT	Catalase
Chl <i>a</i>	Chlorophyll <i>a</i>
cm	centimeters
CO ₂	Carbon dioxide
CPA	Chlorophenoxy acetate
CR	Controlled release
CS	Capsule suspension
CS	Chitosan
DDA	2, 2- bus (p-chlorophenyl) acetic acid
DDD	1,1 dichloro-2,2 -bis (p- chlorophenyl) ethylene
DDMU	1-chloro-2, 2-bis (p-chlorophenyl) ethylene
DDOH	2, 2-bis (p-chlorophenyl) ethanol
DDT	Dichloro diphenyl trichloro ethane
DF	Dry flowable
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DP	Dustable powder
DSC	Differential scanning calorimetry
DW	Dry weight
EC	Emulsifiable concentrate
EE	Encapsulation efficiency
EPA	Environment Protection Agency
et al	And others

etc.	And other things
FESEM	Field emission scanning electron microscopy
FNT	Fenitrothion
FS	Flowable concentrate for seed treatment
FTIR	Fourier transform infrared spectroscopy
g	Gram
g CM ⁻¹	Gram per centimeter
g kg ⁻¹	Gram per kilogram
GC-MS	Gas chromatography-mass spectrometry
g L ⁻¹	Gram per liter
gmol ⁻¹	Gram per mole
GR	Glutathione reductase
GR	Granule
GSH	Glutathione
GST	Glutathione-S-transferase
h	Hour
H ₂ O	Water
H ₂ SO ₄	Sulfuric acid
k	Rate constant
K ₂ HPO ₄	Di potassium hydrogen phosphate
kg	Kilogram
kg. a.i. ha ⁻¹	kilograms of active ingredient per hectare
kg. ha ⁻¹	Kilogram per hectare
KH ₂ PO ₄	Potassium di hydrogen phosphate
LD ₅₀	Median lethal dose
MCP	Monocrotophos
mg g ⁻¹	Milligram per gram
mg kg ⁻¹	Milligram per kilogram
mg L ⁻¹	Milligram per liter
min	Minute

mL	Milliliter
mL ha ⁻¹	Milliliter per hectare
mm	Millimeter
mM	Millimolar
MRL	Maximum residue limit
Mt	Million tonnes
µg	Microgram
µg g ⁻¹	Microgram per gram
µg kg ⁻¹	Microgram per kilogram
µg L ⁻¹	Microgram per liter
µg mL ⁻¹	Microgram per milliliter
µL	Microliter
µm	Micrometer
ngmL ⁻¹	Nanogram per milliliter
NH ⁴⁺	Ammonium
nm	Nanometer
OP	Organophosphorus
Pa	Pascal
PBS	Phycobiliproteins
PC	Phycocyanin
PCP	Pentachlorophenol
PE	Phycoerythrin
POD	Peroxidase
ppb	Parts per billion
ppm	Parts per million
PS I	Photosystem I
PS II	Photosystem II
rpm	Revolution per minute
SC	Suspension concentrate
SDS PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SG	Water soluble granule
SL	Soluble concentrate
SOD	Super oxide dismutase
SP	Soluble powder
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
TPP	Sodium Tripolyphosphate
US	United States
UV	Ultra violet
WG	Water-dispersible granules
WP	Wettable powders
XRD	X-ray diffraction

LIST OF SYMBOLS

%	Percentage
μ	Micron
Da	Dalton
l	Liter
M	Molar
K	kelvin
v/v	Volume by volume
w/v	Weight by volume
β	Beta
$^{\circ}\text{C}$	Celsius
θ	Angle
α	Alpha
γ	Gamma
λ	Lambda

Increased use of agrochemicals due to rising global food demand to feed burgeoning economy, mandates deeper insight on sustainable agricultural production system, which has resulted in greater emphasis on food security and agriculture (Oerke and Dehne, 2004; Shand, 2007). Excessive use of agrochemicals, on the other hand, is a serious impediment to achieving global food security, since it has a negative impact on human health and the environment. Worldwide annual consumption of chemical pesticide has been 4.6 million tons, of which 85% is consumed in the agriculture sector, while the remaining 15% for other purposes (Zhang et al., 2018; Idrovo, 2000). Application of herbicides, insecticides, fungicides and other pesticides in agriculture is 47.5, 29.5, 17.5 and 5.5% respectively (De et al., 2014). Global yearly agrochemical usage is expected to surge by 3.5 million tonnes in coming years (Sharma et al., 2019; Zhang, 2018). Besides benefits of agrochemicals such as crop management to enhance crop productivity and yield, combat plant-related diseases and increase food quality, they are also known for their toxic properties, hence posing a threat to living organisms in the soil and rivers due to losses during their application via degradation, volatilization, photolysis and leaching. Furthermore, only 0.1% of the applied agrochemicals are delivered to the particular target site and act effectively against the target organism (Popp et al., 2013; Camara et al., 2019). Therefore, there is a strong need to identify and investigate new methods of pesticides usage to minimize their application if cannot be stopped.

Rice is the staple food of more than 65% of Indian population (Subbaiah et al., 2001). Biotic stress caused by insect pest's remains as one of the major challenges to maintain economy of agriculture. 10-15 % crop loss is due to plant hoppers, leaf folder gall midge and stem borers. Pesticides are used by the farmers for so many years all over the world. Soil micro flora including cyanobacteria of rice field ecosystem is threatened due to indiscriminate use of these pesticides (Roger et al., 1994).

Cyanobacteria (blue green algae) are integral part of paddy cultivation as it helps in yield improvement, soil quality maintenance and nitrogen fixation (Saadatinia and Riahi, 2009). Both heterotrophs and autotrophs present in soil help to derive native soil nitrogen in paddy field through biological nitrogen fixation (Singh et al., 2013). Cyanobacterial biofertilizer can be considered as boon for paddy field as it is ecofriendly and increases soil fertility. Cartap hydrochloride is organo nitrogen insecticide that is deliberately applied in paddy fields of India, at a recommended dose of 10 $\mu\text{g ml}^{-1}$ to kill chewing and sucking insect pest of rice

crop (Singh et al., 2014). Two types of formulations (4% granules and 50% powder) are available under different trade names (AG-Tap, Vegetox, Padan, Thiobel and Kritap) in India (Kalyaniwala et al., 2016). Due to its high insecticidal activity, it has been used throughout the world (Singh et al., 2014). Cartap's indiscriminate application has resulted in its persistence in the environment due to erosion, leaching, and runoff water from its application sites, which has impacted non-target microorganisms.

Prolonged insecticide use is a major environmental issue, as insecticides possess risk to the environment and human health (Sharma et al., 2020). Unavoidable pesticide use in current agricultural practices has inspired researchers to develop smarter formulations that can minimize use of such hazardous agrochemicals. The use of nanosystems under *in vitro* conditions in medicine and diagnostics has attracted attention in agricultural nanotechnology which enables safe handling of these agrochemicals and is site specific and also released in controlled manner (Kumar et al., 2015).

Biopesticides/natural pesticides are one choice, whereas modern formulations with a limited amount of insecticide (controlled release formulations) are another (Liu et al., 2002). In recent years, there has been a growing interest in nanotechnology as a way to improve the efficacy of insecticides while also reducing their environmental effects (Kumar et al., 2014). The various properties of polymers (biodegradable and biocompatible nature, easy handling) make them appropriate for controlled release of encapsulated material without harming the non-target organism. Numerous methods have been developed to control insects, including biological control, which takes a long time. In this scenario, controlled release formulations (CRFs) have emerged as a very appealing alternative in which active compound is associated with inert materials. These controlled release systems are smart delivery systems that helps in controlling the rate of release of compound into the target site (bioavailability) over a set period of time without interfering the ability of bioactive compound to reach target organism and can also protect non target organisms (Petu et al., 2010). Nanospheres are solid hollow particles with diameter in nanometer range and their empty core posses' great potential for encapsulation of guest molecules (Wilkins, 2004). These nanoparticle insecticide formulations can be readily dispersed in solution which helps in eliminating the problems associated with leaching and drifting. These characteristics allow the use of smaller amount of active compound per area, as long as the formulation may provide an optimal concentration delivery for the target insecticide for longer period of times. They also

minimize cost, reduce phytotoxicity, inflammation of the human mucous membrane, environmental harm to other non-targeted species and even the crops themselves because they don't need to be reapplied (Margulis and Magdassi, 2012). The primary goal of nanoformulations is to release the active ingredient slowly and precisely while also shielding it from premature degradation. Polymers that are biodegradable and biocompatible in nature are appropriate for encapsulation of pesticide for their controlled release to the target organism (Peteu et al., 2010). Natural carbohydrate polymers, such as chitosan (CS), alginate (ALG), pectin etc. are brought into light as these are cost-effective polymers and are biocompatible, biodegradable (Mukhopadhyay et al., 2015).

In Punjab, India cartap hydrochloride is recommended by Punjab Agriculture University, Ludhiana for its use in paddy fields for controlling insect pests and but its repeated application results in buildup of its residues at levels, which are dangerous to the non-target microorganisms including nitrogen fixing cyanobacteria. Considering their importance as biofertilizer in paddy cultivation together with use of cartap mainly to kill stem borer insect pest of rice crop, the present study was undertaken to evaluate physiological activities in *Anabaena variabilis* ARM 441 on exposure to cartap hydrochloride. Furthermore, cartap hydrochloride was chosen as the active ingredient for encapsulation in nanospheres Due to high demand of cartap for a wide range of crops and environmental issues, effort have been made to synthesize cartap nanospheres by encapsulating it within natural polymer, which act as physical barrier and also keep a check on its release. Two types of nanospheres i.e., Alginate–chitosan nanospheres and Chitosan Tpp nanospheres were prepared for controlled release of cartap and to over-come the problems of conventional method of administration of cartap. Nanospheres were characterized by DLS (dynamic light scattering), X-ray diffraction (XRD), field emission scanning electron microscope (FESEM) and Fourier transforms infrared (FTIR) spectra. The *in vitro* release studies were performed using different mathematical models. These nanoformulation potentially hold the key to the development of effective pest and disease management, as they provide site-specific delivery and controlled release of active ingredients thus enhancing efficacy and efficiency. They can also lower the toxicity of agrochemicals by enhancing agrochemical uptake thus improving solubility and stability, as well as help in minimizing problem of volatilization, leaching and run-off resulting in resolving issue of food security and agricultural sustainability.

Rice (*Oryza sativa*) serves as staple food for more than half of the world population (Xu et al., 2016). High-yielding crop cultivars and pesticides usage has considerably increased food production over the past 50 years (Fuad et al., 2012). The most important oxygen evolving photosynthetic prokaryotes i.e. cyanobacteria (blue green algae) are the primary supplier of nitrogen for paddy fields. In the past couple of decades, deliberate use of pesticides has resulted in biomagnification that has negatively impacted soil microbes including cyanobacteria. The abundance of cyanobacterial population due to its mineralizing and nitrogen fixation properties, have enormous potential to mitigate the pesticide negative effects. Ecotoxicological effect of different insecticides on the cyanobacterium have been extensively studied.

2.1 Cyanobacteria

Cyanobacteria are phototrophic prokaryotes also known as “Pond scum” are a group of Gram-negative bacteria (eubacteria) which are filamentous, have nitrogen fixing capability due to presence of heterocyst and enrich the soil with nitrogen and carbon. Morphologically cyanobacteria are classified into five different orders (Fig. 2.1) viz. *Pleurocapsales*, *Chroococcales*, *Oscillatoriales*, *Nostocales* and *Stigonematales* (Rippka et al., 1979; Komarek, 2006).

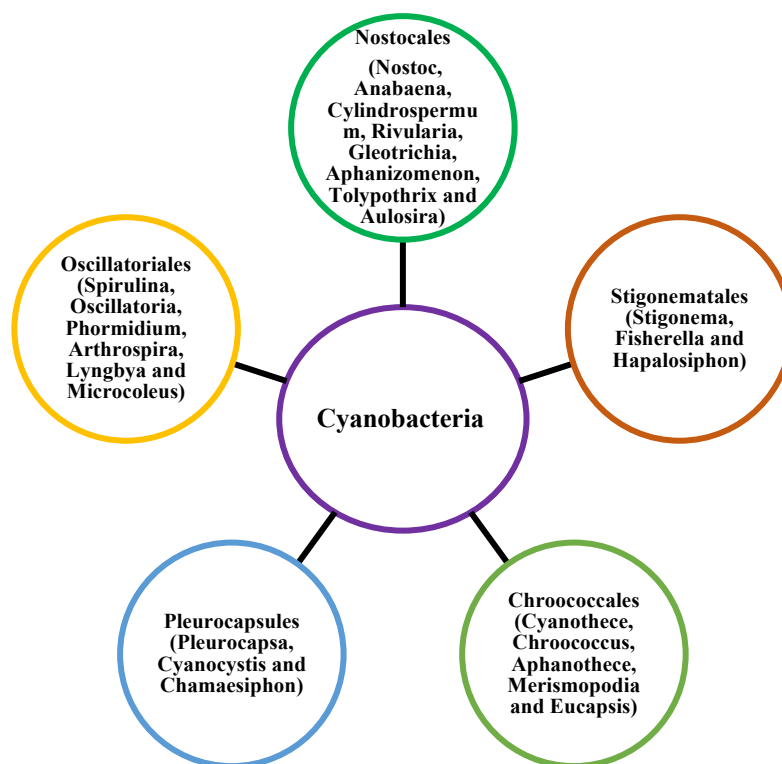


Fig. 2.1: Classification of the Phylum: Cyanobacteria

Judicious use of cyanobacteria can improve soil structure and water holding capacity, organic content, plant stimulating hormones, nitrogen status and extra cellular polysaccharides of soil. The native soil nitrogen pool provides more than half of the total nitrogen utilized in paddy fields which is maintained through biological nitrogen fixation by both heterotrophs and autotrophs in soil. Biological nitrogen fixation is the most common nitrogen fixation process, estimated 1.93×10^8 tonnes of nitrogen is fixed through biological nitrogen fixation each year all over the world (Miyamoto et al., 2008). Cyanobacteria have been applied in rice fields as biofertilizers for improving crop yield (Mishra and Pabbi, 2004; Prasanna and Nayak, 2007; Kaur and Goyal, 2014). The source of biological nitrogen fixation in paddy field is natural population of cyanobacteria (Blue-Green Algae). Cyanobacteria take ammonia by passive diffusion or as ammonium (NH_4^+) by using a specific uptake system (Mishra and Pabbi, 2004). Different species of cyanobacteria (heterocystous and non heterocystous) have been reported from paddy fields of India (Table 2.1), which help in nitrogen fixation. Addition of algal biofertilizer in rice field can increase the yield up to 25-34 % (Chaudhary and Kennedy, 2005; Sao and Samuel, 2015).

Table 2.1 Cyanobacterial diversity reported in paddy fields of India

S. No.	Location	Genus	References
1.	Uttar Pradesh	<i>Phormidium</i> , <i>Anabaena</i> , <i>Nostoc</i> , <i>Lyngbya</i> , <i>Oscillatoria</i> , <i>Hapalosiphon</i> , <i>Gloeocapsa</i> , <i>Calothrix</i> , <i>Westiellopsis</i> , <i>Tolypothrix</i>	Venkataraman, 1975 Prasanna and Nayak, 2007 Srivastava et al., 2009
2.	Kerala	<i>Anabaena</i> , <i>Phormidium</i> , <i>Calothrix</i> , <i>Nostoc</i> , <i>Cylindrospermum</i>	Venkataraman, 1975 Thajuddin and Subramanian, 1992 Srivastava et al., 2009
3.	Meghalaya	<i>Cylindrospermum</i> , <i>Anabaena</i> , <i>Nostoc</i>	Tiwari and Singh, 2005 Nongbri and Syiem, 2012
4.	Assam	<i>Gloeocapsa</i> , <i>Calothrix</i> , <i>Anabaena</i> , <i>Cylindrospermum</i> , <i>Nostoc</i> , <i>Aulosira</i> , <i>Lyngbya</i> , <i>Phormidium</i> , <i>Westiellopsis</i>	Venkataraman, 1975 Yashmin, 2003 Rout and Borah, 2009 Bhardwaj and Baruah, 2013.
5.	Tamil Nadu	<i>Anabaena</i> , <i>Gloeocapsa</i> , <i>Oscillatoria</i> , <i>Hapalosiphon</i> , <i>Scytonema</i> , <i>Lyngbya</i> , <i>Phormidium</i> , <i>Plectonema</i> , <i>Calothrix</i> , <i>Westiellopsis</i> ,	Venkataraman, 1975 Thajuddin et al., 2002 Muthu kumar et al., 2007
6.	Orissa	<i>Phormidium</i> , <i>Nostoc</i> , <i>Hapalosiphon</i> , <i>Anabaena</i> , <i>Calothrix</i>	Sahu et al., 1996 Dey et al., 2010
7.	Bihar	<i>Phormidium</i> , <i>Calothrix</i> , <i>Oscillatoria</i> , <i>Hapalosiphon</i> , <i>Anabaena</i>	Prasanna and Nayak, 2007
8.	Jammu and Kashmir	<i>Westiellopsis</i> , <i>Nostoc</i> , <i>Phormidium</i> , <i>Gloeotheca</i> , <i>Calothrix</i>	Venkataraman, 1975 Bhushan and Kumar, 2013a
9.	Punjab	<i>Calothrix</i> , <i>Anabaena</i> , <i>Nostoc</i> , <i>Cylindrospermum</i>	Venkataraman, 1975 Nayak et al., 2009
10.	Karnataka	<i>Cylindrospermum</i> , <i>Lyngbya</i> , <i>Nostoc</i> , <i>Tolypothrix</i> , <i>Phormidium</i>	Venkataraman, 1975 Thajuddin and Subramanian, 1992

2.2 Rice

Rice belongs to family Poaceae (grass family) is the staple cereal worldwide providing a reliable food source for thirty-nine countries and about half of mankind on the planet (Juliana, 1993). Rice is a dietary energy source that accounts for 22% of total energy consumption globally (Kainuma, 2004). The second highest produced and consumed crop in world is rice and to meet demand of increasing population, its production has to be increased

(Kubo and Purevdoj, 2004). It can be grown in a variety of climates, from 44° north in North Korea to 35° south in Australia, as well as from six feet below sea level (as in Kerala, India) to 2700 feet above sea level in the Himalayas. Rice is best grown in regions of higher humidity and temperature (20 to 37.5° C) with continuous water supply and sunshine (<https://icar-nrri.in/>). Many Asian countries consider the crop to be an important part of their heritage and culture. It is the staple food for more than 65 percent of the Indian population. India cultivates rice in 43 million hectares, producing 112 million tonnes (Mt) of milled rice with an average yield of 2.6 tonnes per hectare. Rice production increased marginally from 1024.86 to 1146.38 lakh metric ton from 2015 to 2020 (Fig. 2.2).

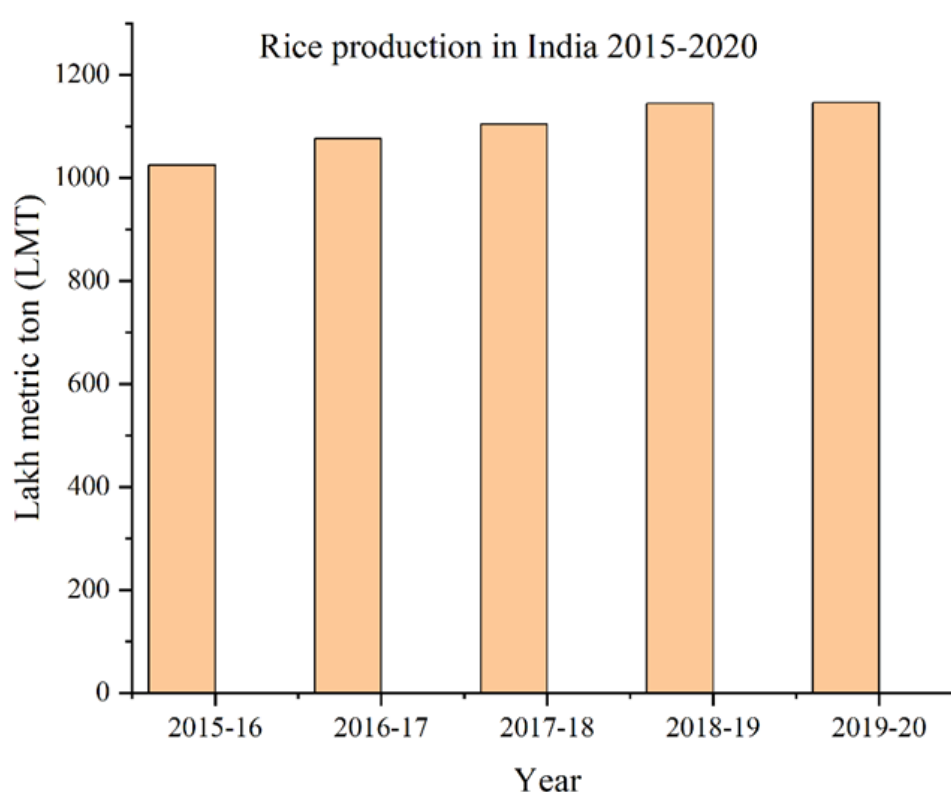


Fig. 2.2: Rice production in India 2015-20

Source: Ministry of Consumer Affairs, Food and Public Distribution
(<https://pib.gov.in/PressReleaseDetailm.aspx?PRID=1697364>)

Global rice production in 2019 reported 491139 thousand metric tons with relatively 0.9 % increase as compared to previous season. China, India, and Indonesia are the top three rice producing countries (Table 2.2) (www.fao.org). These countries also have the highest rice consumption worldwide. Rice consumption in India increased from 98244 kg/hectare in 2014-15 to 100000 kg/hectare in 2018-2019 (Table 2.3).

Table 2.2: Worldwide Rice Production (Kg/Hectare) (2014/15-2018/19)Source: <http://www.airea.net/page/65/world-rice-production-consumption-and-stocks>

Countries	2014-15	2015-16	2016-17	2017-18	2018-19
China	146726	148499	147766	148873	143582
India	105482	104408	109698	112910	111000
Indonesia	35560	36200	36858	37000	37300
Bangladesh	34500	34500	34578	32650	34500
Vietnam	28166	27584	27400	28471	29069
Thailand	18750	15800	19200	20370	20700
Burma	12600	12160	12650	13200	13120
Philippines	11914	11008	11686	12235	12150
Brazil	8465	7210	8383	8208	8160
Japan	8079	7876	7929	7787	7700
Pakistan	7003	6802	6849	7450	7400
Cambodia	4925	4931	5256	5399	5500
Korea, South	4241	4327	4197	3972	3868
Nigeria	3782	3941	4410	4662	4788
Nepal	3190	2863	3480	3310	3530
United States	7106	6133	7117	5659	6931
Others	41672	41977	43363	42910	41841
World Total	482161	476219	490820	495066	491139

Table 2.3: Worldwide Rice consumption (Kg/Hectare) (2014/15-2018/19)Source: <http://www.airea.net/page/65/world-rice-production-consumption-and-stocks>

Countries	2014-15	2015-16	2016-17	2017-18	2018-19
China	141000	141028	141761	142487	142782
India	98244	93568	95776	98660	100000
Indonesia	38300	37850	37800	38000	38100
Bangladesh	35100	35100	35000	35200	35200
Vietnam	22000	22500	22000	22000	22200
Philippines	13000	12900	12900	13250	13500
Burma	10500	10400	10000	10100	10200
Thailand	10000	9100	12000	11000	10500
Japan	8830	8806	8730	8655	8600
Brazil	7925	7900	8000	8000	8025
Nigeria	6100	6400	6700	7100	7400
Korea, South	4197	4212	4651	4883	4673
Egypt	4000	3900	4300	4200	4100
Cambodia	3900	3900	4000	4100	4200
Nepal	3770	3353	4010	3965	4130
United States	4284	3580	4230	4279	4223
Others	61526	62283	65944	66984	68882
World Total	474782	468810	483766	482817	489560

Punjab, West Bengal, and Uttar Pradesh are the top three rice-producing states in India (Table 2.4). During 2019-20, Punjab's rice crop covered 31.42 lakh hectares, yielding 189.18 lakh tonnes of paddy (117.82 lakh tonnes of rice).

Table 2.4: Production of rice in different states of India (lakh metric ton)

Source: Ministry of Consumer Affairs, Food and Public Distribution

(<https://pib.gov.in/PressReleaseDetailm.aspx?PRID=1697364>)

S. No.	State	2015-16	2016-17	2017-18	2018-19	2019-20
1.	Andhra Pradesh	74.89	74.52	81.66	82.35	86.38
2.	Telangana	30.47	51.73	62.62	66.7	73.37
3.	Assam	51.25	47.27	52.84	52.21	50.98
4.	Bihar	68.02	82.39	80.93	61.56	60.53
5.	Chhattisgarh	57.89	80.48	49.31	65.27	65
6.	Gujarat	17.02	19.3	18.91	19.12	19.73
7.	Haryana	41.45	44.53	45.23	45.16	48.24
8.	Jammu and Kashmir	6.46	5.72	5.13	6.16	-
9.	Jharkhand	28.82	38.42	40.78	28.94	31.91
10.	Karnataka	30.21	26.05	30.17	34.31	36.76
11.	Kerala	5.49	4.37	5.21	5.78	6.14
12.	Madhya Pradesh	35.47	42.27	41.24	44.95	48.02
13.	Maharashtra	25.93	31.1	27.31	32.76	31.83
14.	Odisha	58.75	83.26	65.51	77.34	80.37
15.	Puducherry	0.44	0.52	0.43	0.63	-
16.	Punjab	118.23	115.86	133.82	128.22	117.82
17.	Tripura	7.95	8.15	8.12	7.93	-
18.	Tamil nadu	75.17	23.69	66.39	61.31	71.81
19.	Uttar Pradesh	125.01	137.54	132.74	155.45	155.24
20.	Uttrakhand	6.39	6.3	6.47	6.18	6.54
21.	West Bengal	159.54	153.03	149.67	162.42	155.7

2.3 Pesticides and their classification

Pesticide are chemicals used to kill pests and includes herbicides, insecticides, fungicides, and rodenticides, among others. Classification of pesticides is based on their chemical nature e.g., organochlorine, organophosphorus, thiocarbamates, pyrethroids and neonicotinoids (Tiwana et al., 2007).

2.3.1 Pesticides commonly used by farmers in Punjab

In a report by Bhushan et al., (2013b) pesticides which are commonly used by farmers in paddy field are as follows:

1. **Herbicides:** Anilofos, Pendimethelene, Pretilachlor, Sethoxydim, Bensulfuron, Metsulfuron Methyl, Thiobencarb, Oxadiargyl, Ethoxysulfuron, Methyl Butachlor.
2. **Insecticides:** Quinalphos, Carbendazim, Endosulfan, Carbendazim, Chlorpyrifos, Monocrotophos, Cartap hydrochloride
3. **Fungicides:** Carboxin, Copper Oxychloride, Pencycuron, Propiconazole

Pesticides have been reported to hinder cellular functions (photosynthesis, respiration, cell division, development and molecular composition) in a variety of microbial species (De Lorenzo et al., 2001). Therefore pesticides classification is mainly based on their mode of action. For instances, hill reaction and photosystem II is inhibited by triazinones, phenylcarbamates, triazines, urea and acylanilides. Photosystem I is inhibited by paraquat and diquat. Carbamate, organophosphate and organochlorine, primarily target nervous system of insects.

Commonly used insecticides organophosphate (Table 2.5a), Organobromine (Table 2.5b), Pyridine carboxamide (Table 2.5c), Thiazadine (Table 2.5d), Pyrethroid (Table 2.5e), Pyridine (Table 2.5f), Carbamate (Table 2.5g), Neonicotinoid (Table 2.5h), Organofluorine (Table 2.5i), Phenyl pyrazole (Table 2.5j), organochlorine (Table 2.5k), Organo thiophosphate Insecticide (Table 2.5l), Di benzoyl hydrazine (Table 2.5m) and different insecticide combinations (Table 2.5n) and their dosage value, which are used in paddy fields and registered under the Insecticides Act, 1968 up to 30.06.2021 as given by Central Insecticides Board and Registration Committee is compiled in Table 2.5.

Table 2.5: Commonly used insecticides, their dosage value, which can be used in paddy fields a) organophosphate, b) Organobromine, c) Pyridine carboxamide, d) Thiazidaine, e) Pyrethroid, f), Pyridine g) Carbamate, h) Neonicotinoid, i) Organofluorine, j) Phenyl pyrazole, k) organochlorine, l) Organo thiophosphate Insecticide, m) Di benzoyl hydrazine and n) different insecticide combinations; Abbreviations: SP (water soluble powder), DF(dry flowable), SG (water soluble granule), EC (emulsifiable concentrate), DP (Dispersible powder), SC (suspension concentrate), CS (capsule suspension), GR (granule), CG (encapsulated granule), WG (water dispersible granule), DW (dry weight), FS (flowable concentrate for seed treatment), SL (soluble concentrate), WS (water dispersible powder for slurry), AF (aqueous flowable), SE (suspo- emulsion), ZC (mixture of SC AND CS), a.i. (active ingredient).

Source: Central Insecticides Board and Registration Committee (major uses of pesticides) (<http://ppqs.gov.in/divisions/cib-rc/major-uses-of-pesticides>)

a: Organophosphate insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Acephate 75 % SP	Yellow stem borer, Leaf folder, Plant Hoppers, Green leaf hopper	666-100	300-500
Acephate 97 % DF	Yellow stem borer, Leaf folder, Plant hoppers, Green leaf hopper	727.50	750
Acephate 95 % SG	Stem borer, Leaf folder, Brown plant hopper	562.50	592
Chlorpyrifos 10%	Stem borer, Leaf folder, Gall midge	1000	10000
Chlorpyrifos 75%	Stem borer, Leaf Roller, Gall midge	375-400	500-533
Chlorpyrifos 20% EC	Gall midge, Stem borer, Whorl maggot	250	1250
Chlorpyrifos 50% EC	Stem borer, Leaf folder	375-400	750-800
Chlorpyrifos 1.50% DP	Stem borer, Green leaf hopper, Brown plant hopper, Leaf folder, Gall midge, Grass hopper	375	25000
Malathion 5 % DP	Rice Hispa	1250	25000
Malathion 50% EC	Rice Hispa	575	1150

b: Organobromine insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Chlorantraniliprole 18.50%	Stem borer and leaf folder	30	150
Chlorantraniliprole 0.40%	Yellow stem borer, Leaf folder	40	10000

c: Pyridine carboxamide Insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Flonicamid 50 % WG	Brown plant hopper, White backed plant hopper, Green leaf hopper	75	150

d: Thiazadine Insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Buprofezin 25% SC	Brown plant hopper (BPH), Green leaf hopper (GLH), White Back Plant Hopper (WBPH)	200	800
Buprofezin 70% DF	Brown plant hopper	175	250

e: Pyrethroid Insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Bifenthrin 8.80%	Stem borer, leaf folder and Green leaf hopper	44	500
Bifenthrin 10% EC	Stem borer, Leaf folder, Green leaf hopper	50	500
Deltamethrin 11% w/w EC	Stem borer, Leaf folder, Green leaf hopper, Whorl maggot	15	150
Deltamethrin 1.80 % EC	Stem borer, Leaf folder	10 - 12.50	625 - 780
Ethofenprox 10 % EC	Brown plant hopper, Green leaf hopper, Stem borer, Leaf folder, Gall	50 - 75	500 - 750

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
	midge, Whorl maggot, White backed plant hopper		
Fenprothrin 30 % EC	Yellow stem borer, Leaf folder	100	333
Lambda-cyhalothrin 4.90 % CS	Stem borer, Leaf folder	12.50	250
Lambda-cyhalothrin 2.50 % EC	Leaf folder, Stem borer, Green leaf hopper, Gall midge, Hispa, Thrips	12.50	500
Lambda-cyhalothrin 5 % EC	Leaf folder, Stem borer, Green leaf hopper, Gall Midge, Rice hispa, Thrips	12.50	250

f: Pyridine insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Pymetrozine 50 % WG	Brown plant hopper	150	300

g: Carbamate Insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Benfuracarb 3% GR	Stem borer, Leaf folder, BPH	1000	33000
Carbofuran 3% CG	Brown plant hopper Gall midge, Stem borer, Green leaf hopper, Hispa	750	25000
Carbosulfan 6%	Stem borer, Gall midge, Green leaf hopper, Leaf folder	1000	16700
Carbosulfan 25%	Green leaf hopper, White plant hopper, Brown plant hopper	200-250	800-1000
Cartap hydrochloride 4%	Stem borer, Leaf folder, Whorl Maggot	750-1000	18750-25000
Cartap hydrochloride 50%	Stem borer, Leaf folder	500	1000
Cartap hydrochloride 75%	Yellow stem borer, Leaf folder	318 - 375	425 - 500
Fenobucarb 50 % EC	Brown plant hopper, Green leaf hopper	250 - 750	500 - 1500

h: Neonicotinoid insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Acetamiprid 20% SP	Brown plant hopper (BPH)	10-20	50-100
Clothianidin 50 % WG	Brown plant hopper	10 - 12	20 - 24
Dinotefuran 20 % SG	Brown plant hopper	30 - 40	150 - 200
Imidacloprid 70 % WG	Brown plant hoppers, White backed plant hoppers	21 - 24.5	30 - 35
Imidacloprid 48 % FS	Thrips	0.15	0.25
Imidacloprid 30.50 % m/m SC	Brown plant hopper, White backed plant hopper	21 - 26.25	60 - 75
Imidacloprid 17.80 % SL	Brown plant hopper, White backed plant hopper, Green leaf hopper	20 - 25	100 - 125
Imidacloprid 0.30 % GR	Stem borer	0.045	15.0 kg
Thiacloprid 21.70 % SC	Stem borer	120	500
Thiamethoxam 25 % WG	Stem borer, Gall midge, Leaf folder, White backed plant hopper, Brown plant hopper, Green leaf hopper, Thrips	25	100
Thiamethoxam 70 % WS	Thrips, Green leaf hopper	105	150
Thiamethoxam 75 % w/w SG	Green leaf hopper, Brown plant hopper	113	150

i: Organofluorine insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Flubendiamide 20 % WG	Stem borer, Leaf folder	25	125
Flubendiamide 39.35 % m/m SC	Stem borer, Leaf folder	24.0	50.0
Flubendiamide 0.70 % GR	Stem borer	85 - 100	12.14 - 14.28

j: Phenyl pyrazole insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Fipronil 5 % SC	Stem borer, Brown plant hopper, Green leaf hopper, Rice leaf hopper, Rice Gall midge, Whorl maggot, White backed plant hopper	50 - 75	1000 - 1500
Fipronil 0.30 % GR	Stem borer, Brown plant hopper, Green leaf hopper Rice leaf hopper, Rice gall midge, Whorl maggot, White backed plant hopper	50 - 75	16670 - 25000
Fipronil 0.60 % w/w GR	Stem borer and Leaf folder	60	10
Fipronil 80 % WG	Stem borer, Leaf folder	40 - 50	50 - 62.50

k: Organochlorine insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Indoxacarb 15.80 % EC	Leaf folder (<i>Cnephalocrosis medinalis</i>), Green Semilooper, Stem fly	30	200

l: Organo thiophosphate Insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Oxydemeton-methyl 25 % EC	Blue leaf hopper, white leaf hopper	125 -250	500 -1000
Quinalphos 25 % Gel	Brown plant hopper, Leaf folder, Stem borer, Hispa	250	1000
Quinalphos 5 % Granules	Stem borer	750	15000
Quinalphos 20 % AF	Brown plant hopper, Green leaf hopper, Leaf folder, Stem borer	250 - 300	1250 - 1500
Quinalphos 25 % EC	Brown plant hopper, Leaf folder, Stem borer	250-375	1000-1500
Quinalphos 1.50 % DP	Brown plant hopper	300	20000

m: Di benzoyl hydrazine insecticide

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Chlorpyrifos 1.50% DP	Stem borer, Green leaf hopper, Brown plant hopper, Leaf folder, Gall midge, Grass hopper	375	25000

n: Insecticide combination

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Acephate 50 % + Imidacloprid 1.80 % SP	Brown Plant Hopper, Green Leaf Hopper, Stem borer and Leaf folder	518	1000
Acetamiprid 0.40 % + Chlorpyrifos 20 % EC	Stem borer, Brown plant hopper, White backed plant hopper	10 + 500	2.50
Bifenthrin 3 % + Chlorpyrifos 30 % w/w EC	Stem borer, Leaf folder	24 + 240-30 + 300	800 - 1000
Buprofezin 9 % + Acephate 24 % w/w WP	Brown plant hopper	54 + 144	600
Buprofezin 15 % + Acephate 35 % w/w WP	Brown plant hopper, White backed plant hopper	187.5 + 437.5	1250
Buprofezin 20 % + Acephate 50 % w/w WP	Stem Borer, Leaf folder, Brown plant hopper	200 + 500	1000
Buprofezin 22 % + Fipronil 3 % SC	Brown plant hopper	110 + 15	500
Buprofezin 23.10 % + Fipronil 3.85 % w/w SC	Brown plant hopper	173.25 + 28.88	750
Cartap Hydrochloride 50 % + Buprofezin 10 % w/w WP	Yellow stem borer, Brown plant hopper, Leaf folder, Green leaf hopper, White backed plant hopper	480	800
Cartap Hydrochloride 7.5 % w/w + Eamectin benzoate 0.25 %w/w GR	Yellow stem borer (<i>Scirpophaga incertulus</i>)	18.75 + 562.5	7.5
Cartap Hydrochloride 4 % + Fipronil 0.5% CG	Stem borer, Leaf folder	675 - 900	15 - 20

Insecticide	Common name of the pest	Dosage (gm/ha)	
		a.i. (gm)	Formulation (gm/ml)
Chlorpyrifos 50 % + Cypermethrin 5 % EC	Stem borer, Leaf folder	312 + 32- 375+38	625 - 750
Deltamethrin 0.72 % + Buprofezin 5.65 % w/w EC	Brown plant hopper, leaf folder	0.78 + 62.50- 0.94+75.0	1250 + 1500
Dinotefuran 4 % + Acephate 50% w/w/ SG	Brown Plant Hopper and white Backed Plant Hopper	35 + 400	500
Ethiprole 40% + Imidacloprid 40 % WG	Brown plant hopper	37.50 + 37.50	93.75
Fenobucarb 20 % + Buprofezin 5 % w/w SE	Brown plant hopper, Green leaf hopper	400 + 100	2000
Flubendiamide 4 % + Buprofezin 20 % w/w SC	Yellow stem borer, Leaf folder, Brown plant hopper	35 + 175	175 + 700
Flubendiamide 3.50 % + Hexaconazole 5 % w/w WG	Stem borer, Leaf older	35 + 50	1000
Flubendiamide 7.5 % + Kresoxim Methyl 37.5 % w/w SC	Stem borer and Leaf folder	50 + 250	667
Flubendiamide 19.92 % + Thiacloprid 19.92 % w/w SC	Yellow stem borer Leaf folder	60 + 60	250
Fipronil 4 % + Thiamethoxam 4 % w/w SC	Brown Plant Hopper, Green Leaf Hopper and White Backed Plant Hopper	44 + 44	1100
Imidacloprid 6 % + Lambda-cyhalothrin 4 % SL	Stem borer, Hispa, Plant hopper, Gundhi bug	18 + 12	300
Novaluron 5.25 % + Indoxacarb 4.50 % SC	Rice leaf folder (<i>Cnaphalocrosis medinalis</i>)	22.97 + 19.69	437.5
Acetamiprid 0.40 % + Chlorpyrifos 20 % EC	Stem Borer, Brown plant hopper and White backed plant hopper	10 + 500	2.5
Chlorantraniliprole 9.30 % + Lambda- cyhalothrin 4.60 % ZC	Stem borer, Leaf folder and Green leaf hopper	28 – 35	200 – 250
Chlorantraniliprole 0.50 % + Thiamethoxam 1 % w/w GR	Stem borer, Leaf folder, Brown plant hopper, Green leaf hopper	30.0 + 60.0	6 kg/ha

2.3.2 Consumption of pesticides

Pesticide use has tremendously increased in the last two decades, and their presence has become prevalent in marine and freshwater environments. According to Food and Agriculture Organization of United Nation, the utilization of pesticides had been increased from 3058275 to 4122334 ton between 2001 and 2018 globally (fao.org). In cropland system, global total pesticides consumption was equal to 4122334 tonnes in 2018, in which contributions of herbicides was (1216330 tonnes), followed by insecticides (400266 tonnes), fungicides (530095 tonnes) and rodenticides (4333 tonnes) respectively (fao.org). Among different continents, pesticide consumption was highest in America followed by Asia, Europe, Oceania and Africa (Fig. 2.3).

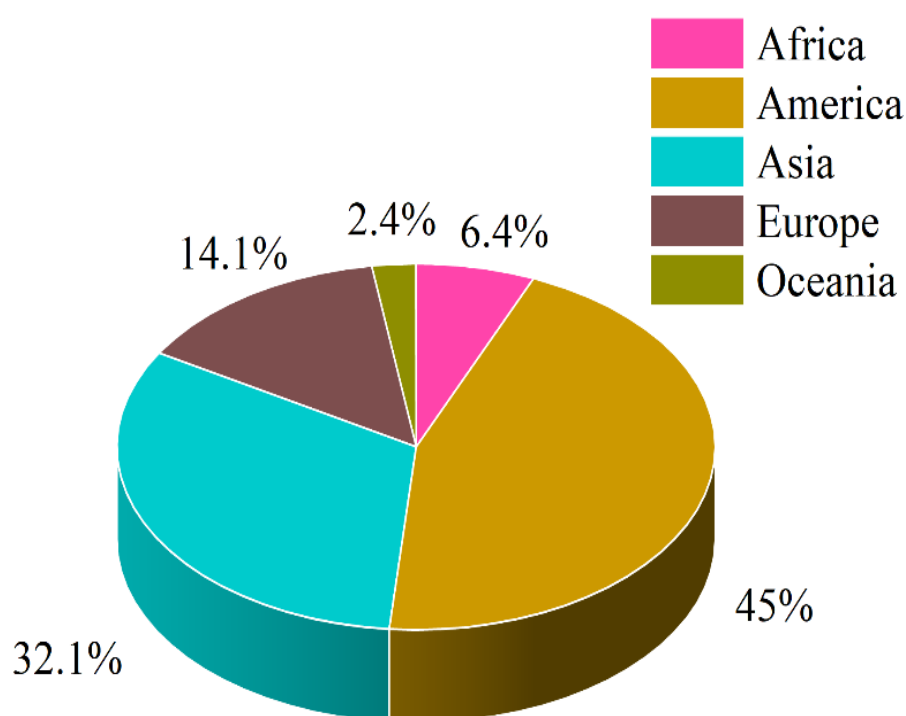


Fig. 2.3: Insecticide usage in different continents

Source: <http://www.fao.org/faostat/en/#data/RP/visualize>

Globally, India is placed at 3rd position in insecticide usage after America and Brazil (Fig. 2.4). In 2020, the consumption of fungicide was highest followed by insecticide, and herbicides were 10318.29, 6668.73, 3296.73 metric tonnes, respectively in 2020 in India (<http://ppqs.gov.in/>).

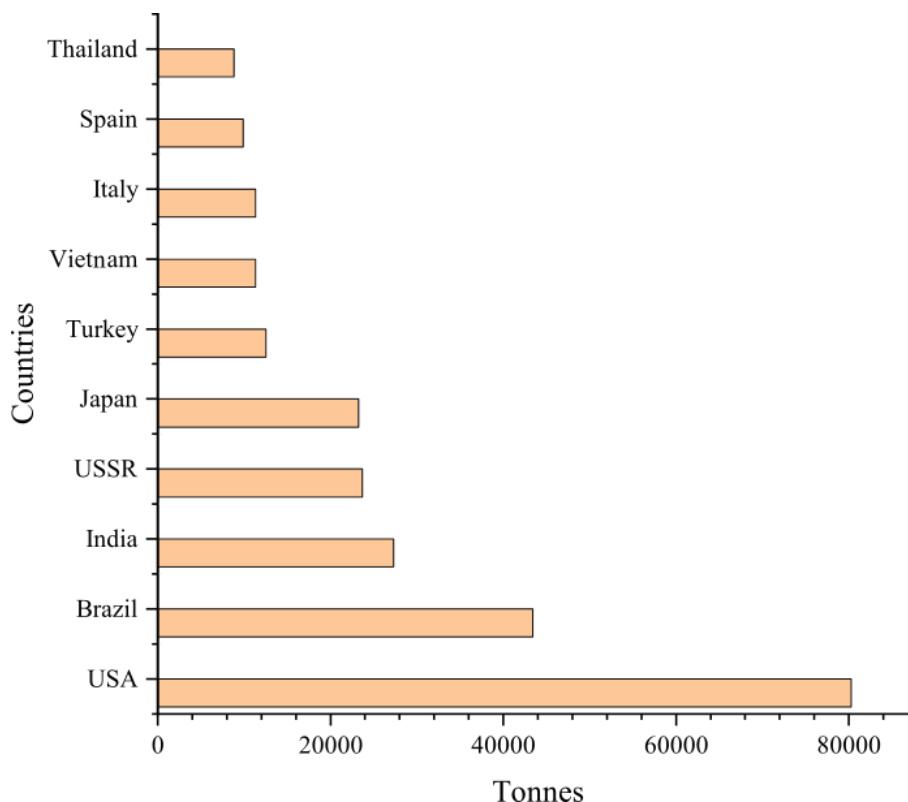


Fig. 2.4: Top ten insecticide utilizing countries

Source: <http://www.fao.org/faostat/en/#data/RP/visualize>

The average pesticides application (kg ha^{-1}) per area of cropland have been increased many folds since 1990s (Roser and Ritchie, 2017). State wise consumption of pesticide revealed that top three pesticides utilizing states in India, are Maharashtra, Uttar Pradesh and Punjab (Table 2.6). Mizoram uses the least amount of pesticides of all the Indian states. In 2016, the application rate was 0.74 kg ha^{-1} in Punjab (Roser and Ritchie, 2017). The consumption of pesticide in Punjab was 5193 metric ton in year 2020 (Table 2.6).

Table 2.6: State wise consumption of pesticides from 2016-2021
Source: Directorate of plant protection, quarantine and storage
(<http://ppqs.gov.in/statistical-database>)

CONSUMPTION OF PESTICIDES IN VARIOUS STATES/UTs						
As on 31.03.2021		Unit: Quantity in Metric Ton Tech. Grade				
S. No.	States/UTs	2016-17	2017-18	2018-19	2019-20	2020-21
1.	Maharashtra	13496	15568	11746	12783	13243
2.	Uttar Pradesh	10614	10824	11049	12217	11557
3.	Punjab	5843	5835	5543	4995	5193
4.	Haryana	4050	4025	4015	4200	4050
5.	Telangana	3436	4866	4894	4915	4986
6.	West Bengal	2624	2982	3190	3630	3630
7.	Rajasthan	2269	2307	2290	2088	2330
8.	Jammu and Kashmir	2188	2430	2459	2198	3352
9.	Tamil Nadu	2092	1929	1901	2225	1834
10.	Andhra Pradesh	2015	1738	1689	1559	1559
11.	Gujarat	1713	1692	1608	1784	1573
12.	Chhattisgarh	1660	1685	1770	1672	1639
13.	Karnataka	1288	1502	1524	1568	1930
14.	Orissa	1050	1633	1609	1115	1158
15.	Kerala	895	1067	995	656	585
16.	Bihar	790	840	850	850	995
17.	Madhya Pradesh	694	502	540	540	691
18.	Jharkhand	541	619	646	681	1161
19.	Himachal Pradesh	341	467	322	881	56
20.	Assam	306	241	256	410	420
21.	Tripura	298	330	349	364	NR
22.	Uttarakhand	198	210	195	224	135
23.	Pondicherry	43	43	42	40	NR
24.	Manipur	33	27	NR	25	46
25.	Goa	22	24	25	30	30
26.	Nagaland	20	20	21	19	36
27.	Arunachal Pradesh	18	NR	5	5	2
28.	Mizoram	9	NR	26	27	NR

2.3.3 Cartap hydrochloride

Cartap is an organo nitrogen insecticide (Fig. 2.5; Table 2.7) and was first used in Japan in 1967 and later introduced in India in 1988 (Singh et al., 2014). Cartap is marketed as ‘AG-Tap’, ‘Vegetox’, ‘Padan’, ‘Thiobel’, and ‘Kritap’ and available in India as two different formulations (4% granules and 50% powder). Cartap due to its high insecticidal activity is used throughout the world for controlling insect pests of paddy, fruit trees, vegetables, tea plants and sugarcane and its mode of action is through stomach. In a rice crop, Jafar et al., (2013) examined the effect of cartap hydrochloride (500 g a.i. /ha) on *Paederus sp.*, predatory coccinellids, *Cyrtorhinus sp.*, spiders and *Microvelia sp.* It is also used to prevent and eradicate cabbage caterpillars in rice and sugarcane fields and the larvae of the Snout Moth. It works as a synaptic blocker and is quickly absorbed by plant tissue. Cartap (nereistoxin) acts by blocking neuromuscular transmission *via* inhibition of post-synaptic nicotinic acetylcholine receptor ion channel resulting in convulsions, respiratory failure and death in extreme cases (De et al., 2014). Multi organ failure and disseminated intravascular coagulation has also been reported as one of the causes of mortality (Kumar et al., 2011). Application of cartap hydrochloride significantly affected the physiological properties of some beneficial soil microorganisms such as *Anabaena variabilis*, *Leptolyngbya foveolarum*, *Pseudomonas striata* and *Azotobacter* CBD15 that are commonly used as biofertilizers. The environmental occurrence of this chemical has been rarely examined and its ecotoxicity has not been extensively investigated (Kim et al., 2008).

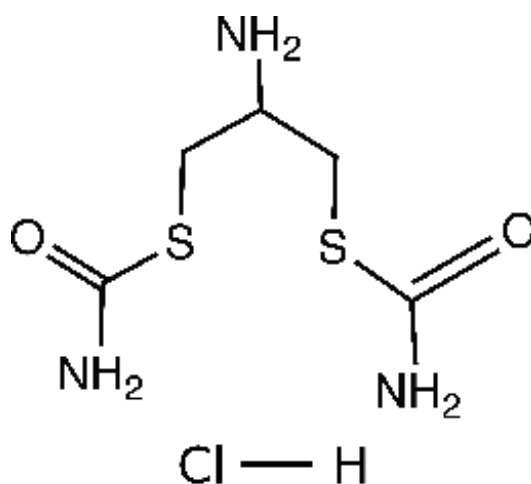


Fig. 2.5: Structure of cartap hydrochloride

Table 2.7: Physicochemical properties of cartap (EPA’s pesticide fact sheet datasheet (1992))

Common name	Cartap Hydrochloride
Trade name	Padan
Chemical class	Organonitrogen
Chemical family	Carbamate
Chemical name	<i>S</i> -[3-carbamoylsulfanyl-2-(dimethyl amino) propyl] carbamothioate; hydrochloride
Molecular formula	C ₇ H ₁₆ ClN ₃ O ₂ S ₂ .HCl
Action	Insecticide
Appearance	Fine powder
Colour	White
Odour	Slightly aromatic odour
Molecular weight	273.66
Melting point	179-181 °C
Solubility	200 g ^l ⁻¹ in water Slightly soluble in methanol and ethanol Hardly soluble in organic solvent
Stability	Stable upto 2 years under field conditions
Mode of field application	Spray and mixed with other pesticides

The consumption of cartap hydrochloride in India from year 2016 to 2021 is given in Fig. 2.6. Cartap is neurotoxic and endocrine disruptor which binds to nicotinic acetylcholine receptors as an open channel blocker. It dreadfully repressed muscarinic acetylcholine receptors of rat which may result in paralysis of muscle and various development deformities (Tomlin, 2000; Eldefrawi and Eldefrawi, 1983; Kwak et al., 2000; Nagata et al., 1997). Cartap cytotoxicity leads to oxidative stress in microorganisms (Liao et al., 2006). India has only a few cases of cartap poisoning (Sumesh and Sheetal, 2014). However, there are considerable knowledge gaps about their environmental impact on non-target microorganisms. Extensive use of cartap in rice fields and due to its high solubility in water (200 g/l), it is very likely to end up in aquatic areas through surface run off and rainfall erosion and may enter food chain causing deleterious effects to humans. The maximum residue limit (MRL) for cartap has been specified by several international organizations around the world. The maximum residue limit for tea and potato is 100 µg kg⁻¹ as specified

by European Commission and Japan. In China, maximum cartap limit 0.1 and 100 $\mu\text{g kg}^{-1}$ was acceptable for rice and sugarcane respectively (Liu et al., 2014).

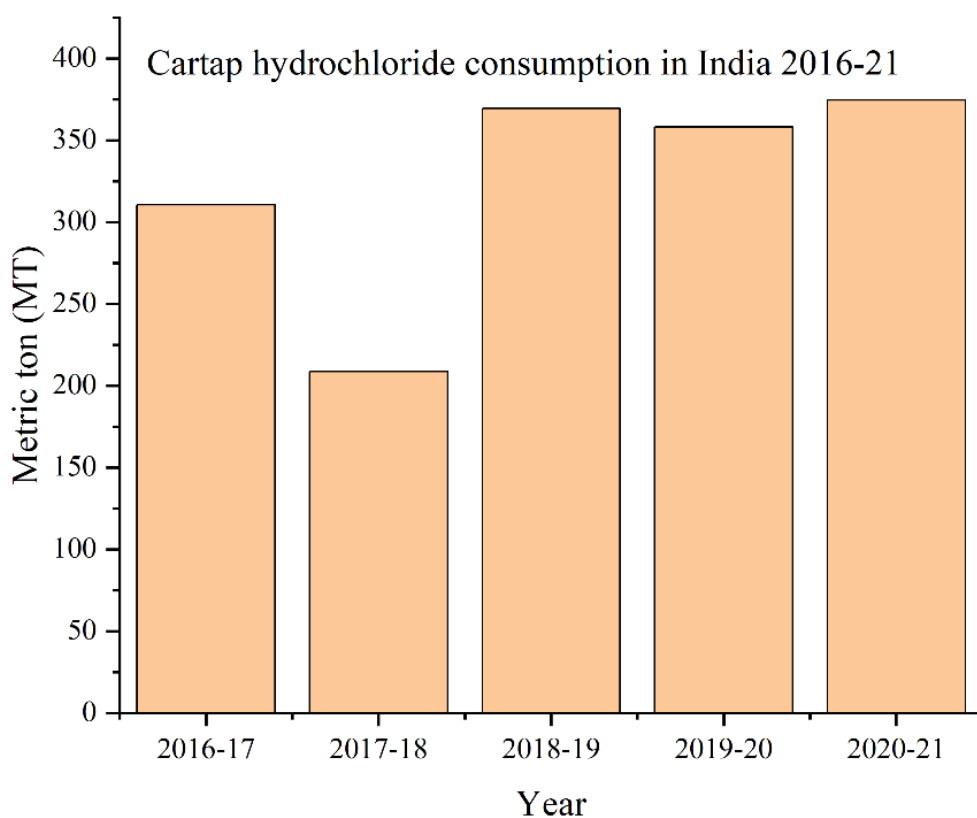


Fig. 2.6: Consumption of cartap hydrochloride in India from 2016-2021

Source: Directorate of plant protection, quarantine and storage

2.3.4 Effect of insecticides on cyanobacteria

Pesticides effects on algae has been thoroughly studied. The following text summarizes the response of cyanobacteria to different categories of insecticides.

2.3.4.1 Carbamate Insecticides

2.3.4.1.1 Carbaryl

Carbaryl is a man-made pesticide that is used to control aphids, fire ants, fleas, ticks, spiders and many other outdoor pests. The growth of *Aulosira fertilissima* and *Nostoc muscorm* was neither inhibited nor stimulated when treated with Sevin at 10 $\mu\text{g ml}^{-1}$ while *Cylindrospermum* sp. showed enhanced growth when treated with same concentration (Ahmed and Vevkataraman, 1973; Padhy and Singh, 1978). Lower concentration of Sevin (0.01 $\mu\text{g ml}^{-1}$) had stimulatory effect on *Westiellopsis prolifica* and *Anabaena* sp. but higher concentration (0.05 $\mu\text{g ml}^{-1}$) inhibited the growth and nitrogen fixation (Adhikary et al.,

1984). Zectran at $0.5 \mu\text{g ml}^{-1}$ affected motility of *Oscillatoria*, at $10 \mu\text{g ml}^{-1}$ suppressed the growth of *Oscillatoria terebriformis* and *Synechococcus lindus* but respiration and photosynthesis were affected at $12.5 \mu\text{g ml}^{-1}$ (Snyder and Sheridan, 1974). $120 \mu\text{g ml}^{-1}$ of carbaryl ruptured the cell wall of *Nostoc muscorum* leading to cell lysis (Vaishampayan, 1984).

Carbamate insecticide aldicarb along with fifteen other insecticides were tested on cyanobacterium *cyanophage* LPP-1 and *Plectonema boryanum* IU 594 by performing disk assays, growth curves, spot tests and one-step growth experiments. Atrazine, fluometuron, ametryn and DCMU inhibited the growth of *Plectonema boryanum* IU 594 whereas declined pattern of growth was observed in *cyanophage* LPP-1 when exposed to isotox and malathion (Mallison and Cannon, 1984).

Toxicity of carborfuron and carbaryl was compared in nitrogen fixing cyanobacterium *Anabaena* PCC 7120 and observed that lethal dose for carborfuron was 80 and $100 \mu\text{g ml}^{-1}$ and for carbaryl was 100 and 200 under nitrogen and non-nitrogen fixing conditions respectively (Padhy and Mohapatra, 2001).

Bhunia et al. (1993) investigated different physiological parameters (hydrogen peroxide, glutathione reductase (GR), superoxide dismutase (SOD), glutathione content) of *Nostoc muscorum* treated with $10 \mu\text{g ml}^{-1}$ of carbaryl resulted in increased SOD and GR activity while decreased glutathione content. In another study by Bhunia et al. (1994), carbaryl ($5\text{-}50 \mu\text{g ml}^{-1}$) inhibited nitrogen assimilating enzymes (nitrate reductase, glutamine synthetase, and nitrogenase), photosynthesis and growth of *Nostoc muscorum* in dose dependent manner but respiration rate increased with increase in carbaryl concentration indicating that respiratory electron transport chain of the microorganism remained unaffected.

No inhibition in growth was observed in four different cyanobacterial species *Aphanizomenon flos-aquae*, *Oscillatoria* sp., *Anabaena inaequalis* and *Pseudanabaena* sp. when treated with $3 \mu\text{g ml}^{-1}$ of carbaryl (Peterson et al., 1994). It was reported that application of carbaryl to soil under flooded was more toxic than non flooded conditions but when hydrolysis product of carbaryl (1-naphthol) was applied to soil, it resulted in increase in cyanobacterial population in soil under both conditions (Megharaj et al., 2011).

In non heterocystous cyanobacterium *Oscillatoria pseudogeminata*, treatment with 100-1000 $\mu\text{g ml}^{-1}$ carbaryl inhibited lipid, protein, carbohydrate, photosynthetic pigments, amino acids and growth in dose dependent manner (Bakiyaraj et al., 2014).

Toxicity of insecticide was induced on cyanobacterial osmolytes, photosynthetic pigments, and antioxidant enzymes when *Calothrix brevissima* was treated with 10-40 $\mu\text{g ml}^{-1}$ of carbaryl for 21 days. A higher concentration of carbaryl (40 $\mu\text{g ml}^{-1}$) inhibited protein, phycobilins and chlorophyll content but stimulated osmolytes, carbohydrate, antioxidant enzymes and malondialdehyde content. Increase in lipid peroxidation indicated free radical mediated deleterious effects of cartap while increase in antioxidant enzymes suggested their role in free radical scavenging. Increased osmolyte concentration might be another adaptive defence strategy of cyanobacterium against insecticide toxicity by cyanobacteria (Habib et al., 2011). Sulfur metabolism, fatty acid profile and electrolyte leakage was studied in *Calothrix brevissima* exposed to 40 $\mu\text{g ml}^{-1}$ of carbaryl was studied which concluded that membrane leakage was increased by 27% while polyunsaturated acids were decreased by 32 % suggesting that lipid peroxidation is caused by free radicals. Elevated levels of sulfur containing amino acids (52-79%) (methionine, cystine and cysteine), non-enzymatic antioxidants (56-72%) and osmolytes (98-179%) suggested their role in mitigating carbaryl stress (Habib et al., 2013).

2.3.4.1.2 Carbofuran

Growth of *Nostoc* and *Cylindrospermum* was promoted when grown in liquid medium supplemented with 25 $\mu\text{g ml}^{-1}$ of carbofuran under nitrogen fixing conditions but fatal at 1500 and 2000 $\mu\text{g ml}^{-1}$ respectively. When these strains were grown in agar plates in nitrogen free medium and nitrogen supplemented media, they were unable to survive upto 300 $\mu\text{g ml}^{-1}$ of carbofuran (Panigrahy, 1984).

Rath and Adhikary (1994) investigated toxicity of furadan against 22 cyanobacterial species and observed that unsheathed cyanobacterial species were insecticide resistant making them perfect to be used as biofertilizers.

Rice field cyanobacterium *Nostoc muscorum*, *Chlorella vulgaris* and *Nostoc linckia* were assessed for carbofuran toxicity. *Nostoc Linckia* was more resistant too insecticide than *C.*

vulgaris and *Nostoc muscorum*. Microscopy studies revealed insecticide interference with cell resulting in membrane abnormalities (Megharaj et al., 1993).

Treatment of carbofuran to *Anabaena doliolum* made it more sensitive due to its sheathless heterocystous form. Low doses of insecticide were tolerated by microorganism but higher dose showed inhibitory effect and proved toxic to microorganism. When cyanobacterium was treated with 100 µg ml⁻¹ of carbofuran, nitrogenase activity was lowered by 38% after 48 h of treatment but heterocyst frequency remained unaffected (Hammouda, 1999).

Fresh water alga *Phormidium fragile* showed no effect on growth when treated upto 112.5 µg ml⁻¹ concentration of methomyl but at high dose gradually decreased photosynthetic pigments, carbohydrate and nitrogen content. The alga was able to metabolize 48.6% of radiolabelled insecticide when grown in its presence (Khalil and Mostafa, 1986).

Five green alga (*Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Scenedesmus obliquus*, *Scenedesmus quadricauda*, and *Selenastrum capricornutum*) and three cyanobacterial species (*Microcystis aeruginosa*, *Microcystis flos-aquae*, and *Anabaena flos aquae*) were examined for toxic effects for five Carbamate insecticides. Toxicity profile of these pesticide to cyanobacteria and green alga was as follows: carbaryl > carbofuran, propoxur, metolcarb > carbosulfan (Ma et al., 2006).

2.3.4.2 Pyrethroid insecticides

The pyrethroid insecticide permethrin and its breakdown products were tested for their toxic effects on the photosynthesis, growth and N₂ fixation capabilities of three cyanobacterial and two green algae species. Except for the cyanobacterium *Anabaena inaequalis*, permethrin was largely non-toxic to all of them, with EC₅₀ values ranging from 1.5 to 5.0 µg ml⁻¹. 3-phenoxybenzaldehyde and 3-phenoxybenzyl alcohol were the most hazardous metabolites of permethrin, followed by benzoic acid, 3-hydroxybenzoic acid, and 3-phenoxybenzoic acid. These compounds had EC₅₀ values as low as 1.4. Green algae was more resistant to these metabolites than cyanobacteria. Different combinations of Permethrin and metabolites were investigated against *Anabaena inaequalis* and found to have antagonistic, synergistic and additive interactions (Stratton and Corke, 1982a, b).

Cypermethrin at two different concentrations of 20 and 50 μM significantly reduced photosynthetic pigments of diazotrophic cyanobacterium *Anabaena doliolum*. Phaeophytin a, as well as fluorescing and non-fluorescing chlorophyll catabolites, were generated during the pigment degradation process (Mohapatra et al., 2003a).

Anabaena sp. PCC 7119 and its resistant strain was evaluated against insecticide cypermethrin through photosynthetic pigment fluorescence. The resistant strain showed minimum change in stress indicating fluorescence parameters (net rate of PS-II closure, energy dissipation of active reaction center and relative variable fluorescence at J level) as compared to normal strain (Chandrakala and Mohapatra, 2012).

Fatma et al. (2007) studied impact of different pollutants (salt stress, pesticide and heavy metal) on *Westiellopsis prolifica* reported reduction in the synthesis of photosynthetic pigment (chlorophyll a) while increased levels of proline was observed when treated with two different insecticides deltamethrin and alphasmethrin.

Singh et al. (2009) investigated the distribution pattern of cyanobacteria in a pesticide-treated rice field. Five different pesticides endosulfan, alachlor, fipronil, carbofenthrin and phorate were employed over the period of study and found that application of every pesticide altered the distribution pattern of cyanobacterial species. During the initial phases of the rice crop's life cycle, non-heterocystous and unicellular forms predominated, while heterocystous forms emerged later. *Oscillatoria late-virens*, *Oscillatoria formosa*, *Chroococcus indicus*, *Synechocystis pevalekii* and *Synechococcus elongatus* were present in all samples collected at different time intervals of rice crop cycle indicating their resistance to insecticides. The frequently found cyanobacterial strains such as *Tolypothrix*, *Calothrix* and *Aulosira* were absent in all samples suggesting their elimination from rice field ecosystem due to deliberate insecticide use.

The effect of insecticide acetamiprid on photosystem II of *Synechocystis* sp. was investigated by using *in vivo* chlorophyll fluorescence. Amount of inactivated PS-II centres increased on exposure to insecticide which resulted in loss of active centres (PS-II_A). The acceptor site of electron transfer was the target location of acetamiprid toxicity in *Synechocystis* sp. PS-II (Li et al., 2010).

2.3.4.3 Organochlorine insecticides

2.3.4.3.1 Dichloro diphenyl trichloro ethane (DDT)

The first modern insecticide widely used to control wide range of insects by acting on nervous system was DDT (1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl) ethane) (Thuy, 2015). However deliberated use of DDT in agriculture resulted in ecosystem damage which resulted in ban on using DDT in developed countries. Because of its profitable and broad-spectrum activity, it is still utilized in several impoverished countries (Mazlan et al., 2017). Previous study reported that DDT usage had resulted in change in composition of marine microalgae (Megharaj et al., 1999a) and also poses hazard to various algal species such as *Anabaena cylindrica*, *Microcystis aeruginosa* (Vance and Drummond, 1969); *Nostoc strains*, *Anabaena variabilis* (Hutber et al., 1979); *Synechococcus elongates* (Worthen, 1973).

Anacystis was the first cyanobacterium to be found to metabolize DDT (Lazaroff and Moore, 1966; Moore et al., 1968). Stunted growth was observed in *Anacystis nidulans*, *Microcystis aeruginosa* (Gregory et al., 1969) and *Anabaena cylindrica* (Vance and Drummond, 1969) when treated with 0.1 $\mu\text{g ml}^{-1}$ of DDT. At concentration of 0.99 $\mu\text{g ml}^{-1}$ of DDT, no deleterious effect was seen on growth and morphology of *Synechococcus elongates* (Worthen, 1973). Axenic cultures of *Microcystis aeruginosa*, *Anacystis nidulans* and *Anabaena cylindrica* intracellularly accumulated 230, 849 and 268 times insecticide from nutrient medium supplemented with 1 $\mu\text{g ml}^{-1}$ of DDT (Vance and Drummond, 1969, Gregory et al., 1969). Worthen (1973) reported that cyanobacterium with 0.1 $\mu\text{g ml}^{-1}$ DDT that was metabolized to DDE (1, 1-dichloro-2, 2-bis (4 chlorophenyl) ethylene).

The persistence of DDT and its metabolites and their effect on cyanobacteria was studied under laboratory conditions for 45 days and results suggested that microalgae in non-sterile soils removed DDE and DDD (1, 1 dichloro-2,2 -bis (p- chlorophenyl) ethylene) by less than 1%. The metabolites DDA (2, 2- bus (p-chlorophenyl) acetic acid) and DDMU (1-chloro-2, 2-bis (p-chlorophenyl) ethylene) was removed in range of 4-8%. During this period 60% of DDOH (2, 2-bis (p-chlorophenyl) ethanol) was degraded and DDT was not found. High concentration (50 or 100 mg kg^{-1}) of DDT, DDE, DDMU and DDOH had no effect on algae because of their poor availability. This may be due to fact that considerable fraction of DDT residues remained bonded to soil components but in very low amount than added in soil (Boul, 1996). However, presence of DDD or DBP significantly reduced algal population. The

toxicity profile of DDT and its metabolites to soil algae followed the order: $DDT \leq DDE \leq DDMU \leq DDOH < DDA < DDD < DBP$ (Megharaj et al., 1999a).

Five axenic cultures were isolated from DDT contaminated soil and screened for cyanobacterial toxicity against metabolites of DDT. 25 % degradation of DDT to DDD was observed in *Nostoc sp.* isolated from PCP (pentachlorophenol) polluted field. In case of *Anabaena sp.*, two metabolites DDE (minor) and DDD (major) were formed (Megharaj et al., 2000).

2.3.4.3.2 BHC

BHC (Benzene hexachloride; a stereoisomer of 1, 2, 3, 4, 5, 6 - hexachloro cyclohexane) has been used worldwide since 1940 to combat insect. Four isomers of BHC (alpha, beta, gamma and delta) which are common constituent of technical products are persistent in nature and accumulated in very high amount in aquatic ecosystem (Sahu et al., 1990). *Microcystis aeruginosa* showed inhibited growth by 50% when exposed to 16% BHC (Fitzgerald et al., 1952). Ishizawa (1966) and Palmer and Maloney (1955) witnessed stimulated growth in cyanobacterial species when isomeric forms of BHC were applied. In contrast, reduction in growth was reported in cyanobacteria after application of gamma BHC application (Sardeshpande and Goyal, 1982). When gamma form of BHC was applied at 10 times more dose than recommended, they enhanced growth of paddy field cyanobacteria (Raghu and Mac Rae, 1967). BHC was tolerated by *Cylindrospermum sp.*, *Plectonema boryanum*, and *Aulosira fertilissima* upto $55 \mu\text{g ml}^{-1}$. *Cylindrospermum sp.* seems to be the most sensitive than other two (Singh, 1973). In laboratory conditions, *Anabaena aphanizomenoides* and *Anabaenopsis raciborskii* detoxified BHC with in ten days (Das, 1977). In another study by Das and Singh (1978) γ -BHC at $40 \mu\text{g ml}^{-1}$ was algistatic to *Anabaena oopsis raciborskii* while at $50 \mu\text{g ml}^{-1}$ it was algicidal to *Anabaena aphanizomenoides*. Under high salinity conditions, *Nostoc calcicola* and *Anabaena variabilis* degraded hexachlorocyclohexane within four months of incubation (Kadirova et al., 2012).

2.3.4.3.3 γ -hexachlorocyclohexane (Lindane)

In a laboratory-grown culture, lindane at $5 \mu\text{g ml}^{-1}$ suppressed the growth of *Aulosira fertilissima* and *Anacystis nidulans*, but no effect was seen in rice fields (Lazaroff and Moore, 1966; Ahmed and Venkataraman, 1973). In *Anacystis nidulans*, high concentration

(10 µg ml⁻¹) of lindane inhibited cell number, photosynthetic pigments and dry weight but its lower concentration stimulated dark respiration and photosynthesis (Kopeck et al., 1976). Sharma (1986) isolated different cyanobacterial species from paddy field showing different tolerance level to lindane. *Aulosira fertilissima* tolerated upto 15 µg ml⁻¹ followed by *Nostoc sp* and *Anabaena doliolum* which tolerated upto 10 µg ml⁻¹ and 9 µg ml⁻¹ respectively.

Lindane was degraded by *Nostoc ellipsosporum* and *Anabaena sp.* strain PCC 7120 into 1, 2, 3 trichlorobenzene (minor metabolite) and 1, 2, 4 trichlorobenzene (major metabolite) (Kuritz and Wolk, 1995). Nitrogen metabolism of cyanobacterium *Anabaena sp.* strain PCC 7120 plays important role in dechlorination of lindane. Experiments conducted using mutant strains of *Anabaena sp.* lacking nitrate utilization system indicated that lindane dechlorination requires operative nir operon (enzyme for nitrate utilization). Similar results were reported in cyanobacterium *Nostoc ellipsosporum* (Kuritz et al., 1997).

Other cyanobacterial strains that have been reported to breakdown lindane include *Calothrix sp.* ATCC 29112, *Nostoc sp. G*, *Nostoc parmelloides* U71627, *Fischerella sp.* CALU926, *Anabaena sp.* 30, *Fischerella muscicola* UT1829, *Nostoc muscorum* UT 387, *Nostoc parmelloides* U71627, and *Nostoc sp.* GSV39 with varying degradation potential (Kuritz et al., 1997). In cyanobacterium *Anabaena*, all physiological parameters (biomass, growth, chlorophyll, protein and starch content) were affected when treated with 0.5- 2.0 µg ml⁻¹ of lindane (Babu et al., 2001).

The stress adaptation strategy of nitrogen-fixing cyanobacterium *Anabaena* PCC 7119 to increasing concentrations of lindane was assessed using polyphasic chlorophyll *a* fluorescence increases measurements. Lower concentration of lindane boosted photosynthetic activity of cells but at higher concentration due to non-competitive inhibition of PS-II, these activities got suppressed. Furthermore, adverse effects on thylakoid of treated cells were confirmed by electron microscopic studies (Bueno et al., 2004).

Six different species of cyanobacteria (*Microcystis*, *Synechococcus*, *Nostoc*, *Nodularia*, *Oscillatoria* and *Cyanothece*) was isolated from Egyptian Lakes Mariut and Qaroun and assessed for lindane degradation. The elimination of lindane by species of Qaroun lake was higher than that of Mariut lake species, which ranged from 71 to 99 percent at 5 µg ml⁻¹ of lindane concentration (El-Bestawy et al., 2007).

2.3.4.3.4 Pentachlorophenol (PCP)

The, growth rate, chlorophyll *a* and algal distribution of Nile River microalga such as *Cyanophyta* (blue-green algae), *Bacillariophyta* (diatoms) and *Chlorophyta* (green algae) were found to be significantly affected with increased concentration of PCP (El-Dib et al., 2000).

Assessment of effect of heavy metals (Na_3PO_4 , Cd^{2+} and Cu^{2+}) and pH was done on biomass collected from natural cyanobacterial bloom for sorption of PCP which revealed that in pH range of 3.25-9, PCP sorption decreases significantly. In case of Cu^{2+} , PCP sorption increases in dose dependent manner in presence of Cd^{2+} . Under the influence of Na_3PO_4 , PCP sorption increases rapidly. Ionic pairing between metal and PCP facilitated its sorption which was solely dependent on pH as confirmed by FTIR and UV visible spectrograms (Tao et al., 2012).

When PCP ($0.001\text{-}10\ \mu\text{g ml}^{-1}$) was supplemented in co culture of green microalga *Chlorella vulgaris* and cyanobacterium *Microcystis aeruginosa* for ten days, at low concentration of PCP increased growth of cyanobacterial community was observed than green alga (Moraes et al., 2014).

2.3.4.3.5 Aldrin, Dieldrin and Endrin

Microcystis aeruginosa was showed stunted growth even at less than $5\ \mu\text{g ml}^{-1}$ of aldrin, dieldrin and endrin (Clegg and Koevenig, 1974). In *Anacystis nidulans* and *Agmenellum quadruplicatum*, the growth was declined when exposed to Dieldrin in range of $0.475\text{-}0.950\ \mu\text{g ml}^{-1}$ (Batterton et al., 1971). Another study by Kopeck et al. (1976) revealed that *Anacystis nidulans* exposed to dieldrin adversely affected photosynthetic pigments, biomass and photosynthesis. At $100\ \mu\text{g ml}^{-1}$, dieldrin and aldrin were proved very toxic to *Nostoc muscorum*, *Anabaena cylindrica* and *Anabaena nidulans* (Schauberger and Wildman, 1977a).

In a study by Vance and Drummoud (1969), cyanobacteria accumulated organochlorine insecticides (endrin, aldrin and dieldrin). *Microcystis aeruginosa* and *Anabaena cylindrica* accumulated 150 to 220 times the $20\ \mu\text{g ml}^{-1}$ of endrin, aldrin and dieldrin supplemented in the medium (Dierksheide and Pfister, 1973).

In cyanobacterium *Nostoc linckia*, treatment of aldrin along with basalin and 2, 4-D suppressed L-methionine-DL-sulfoximine (MSX)-induced photoproduction of ammonia. Except at low concentrations (100 and 500 $\mu\text{g ml}^{-1}$) of 2, 4-D which increased NH_4^+ synthesis, pesticide-MSX combination showed inhibitory nature (Mishra et al., 1989).

When *Anabaena variabilis* (BTA-21) was exposed to aldrin, it was found that lower concentrations had no effect on growth and nitrogen fixing ability but higher concentration proved inhibitory (Nilima and Tiwari, 2001). In another study, *Anabaena sp.* PCC 7120 metabolized aldrin to dieldrin via epoxidation mechanism involving cytochrome P450-dependent monooxygenase in liquid medium (Park and Lee, 2010).

Growth of rice field cyanobacterium *Aulosira fertilissima* was inhibited by 10 $\mu\text{g ml}^{-1}$ of endrin (Ahmad and Venkataraman, 1973). 950 $\mu\text{g ml}^{-1}$ of endrin suppressed growth of *Agmenellum quadruplicatum* while no effect on growth was seen in *Anacystis nidulans*. When these two cyanobacterial species was exposed to 950 $\mu\text{g ml}^{-1}$ of photoaldrin and aldrin, stunted growth was observed (Batterton et al., 1971). However, under lab conditions, 60 $\mu\text{g ml}^{-1}$ of endrin was easily tolerated by *Plectonema boryanum*, *Cylindrospermum sp.* and *Aulosira fertilissima* (Singh, 1973).

2.3.4.3.6 Endosulfan

The growth of *Hapalosiphon intricatus*, *Calothrix bharadwajae* and *Anabaena iyengarii* was significantly reduced after treatment with endosulfan (Sardeshpande and Goyal, 1982). Growth of *Aulosira fertilissima* and *Anabaena sp.* was inhibited by 37.7% and 51.7% on exposure to 1 $\mu\text{g ml}^{-1}$ of endosulfan (Tandon et al., 1988; Lal and Lal, 1988) while in *Westiellopsis prolifica*, 100 $\mu\text{g ml}^{-1}$ of endosulfan suppressed its growth (Adhikary, 1989). A report by Rao and Lal (1987) indicated accumulation of endosulfan by *Aulosira fertilissima* and *Anabaena sp.* and their conversion into endosulfan lactone and endosulfan ether.

Growth of cyanobacterium *Anabaena doliolum* was completely inhibited at 3 $\mu\text{g ml}^{-1}$ when grown in medium supplemented with 0.1 -100 $\mu\text{g ml}^{-1}$ of endosulfan while in *Chlorella vulgaris* (green alga) complete inhibition occurred at 100 $\mu\text{g ml}^{-1}$ of endosulfan. The lethal dose for *Chlorella* and *Anabaena* was 41.5 and 2.15 $\mu\text{g ml}^{-1}$ respectively (Mohapatra and Mohanty, 1992).

The effect of endosulfan was assessed on *Synechococcus cedrorum* (wild and chemically mutated) in terms of glycogen content, growth, photosynthetic pigment synthesis and inorganic nitrogen source uptake (ammonia, nitrate and nitrite). Both the strains showed same pattern growth due to stress generated by endosulfan. Lag phase of both strains increased in dose dependent manner. Paradoxically, uptake of ammonia, nitrate and nitrite along with glycogen accumulation increased with increasing endosulfan concentration. The sole variation in both the strains was the amount of endosulfan that mutant strain tolerated 2.5 times more dose than wild strain (Bisen and Mathur, 1993).

Impact of endosulfan, atrazine and chlorpyrifos was studied on estuarine microbial food web containing cyanobacterial species. With 0.001-0.01 $\mu\text{g ml}^{-1}$ of endosulfan and chlorpyrifos, chlorophyll *a* and cyanobacterial phototropic process was inhibited. With increasing dose of endosulfan *Oscillatoria* and *Melosira* were vanished from ecosystem (De Lorenzo et al., 1999). In wild and mutant *Anabaena flos-aquae*, endosulfan inhibited total nitrogen content, photosynthetic pigments and growth in same way but Mutant strain was more adaptive than wild strain (Shehata et al., 2001).

Biodegradation of endosulfan was observed in two nitrogen fixer cyanobacterial strains *Anabaena flos-aquae* and *Anabaena sp.* PCC 7120 producing endosulfan hydroxyl ether, endosulfan lactone, endosulfan diol and endosulfan sulphate as metabolites after biodegradation. β endosulfan was synthesized in small amount after 24 h of application of α endosulfan, implying that isomerase enzyme was involved (Lee et al., 2003).

Westiellopsis prolifica and *Nostoc hatei* were also reported to metabolize endosulfan to endosulfan sulfate by decreasing insecticide concentration to 40 and 50% respectively when incubated in 2.5 $\mu\text{g ml}^{-1}$ of endosulfan (Jha and Mishra, 2005).

In a report by Prasad et al. (2005), endosulfan treatment in range of 5-20 $\mu\text{g ml}^{-1}$ had significant effect on physiological and biochemical parameters of *Plectonema boryanum*. PS-I and PS-II activity was severely affected. Synthesis of photosynthetic pigments and growth was affected in dose dependent manner. Increased rate of membrane leakage and lipid peroxidation was observed due to formation of active oxygen species resulting in activation of antioxidant defence system.

Oxidative stress response caused due to endosulfan was studied in terms of antioxidant enzymes, biochemical parameters and lipid peroxidation in three cyanobacterial species *Nostoc muscorum*, *Aulosira fertilissima* and *Anabaena variabilis*. Reduction in growth, carbohydrate and photosynthetic pigments was observed along with increase in antioxidant enzymes (catalase, superoxide dismutase and ascorbate peroxidase), proline content and malondialdehyde content. *Nostoc muscorum* showed more pronounced effects followed by *Anabaena variabilis* and *Aulosira fertilissima* (Kumar et al., 2008). Kumar et al. (2009) found that photosynthetic pigments, amino acids, phenols, carbohydrates, glutamine synthetase and nitrate reductase were affected when *Anabaena fertilissima* was treated with 3-12 $\mu\text{g ml}^{-1}$ of endosulfan. Photosynthetic pigments carotenoids and chlorophyll *a* were suppressed by 20 and 38% while phycobiliproteins phycocyanin, phycoerythrin and allophycocyanin were inhibited in range of 28-64% after 96 h of treatment with endosulfan. Enzyme activities were decreased by 3-90%.

Abdel-Raouf and EI-Shafey (2009) reported continuous inhibition in filamentous cyanobacterial species in soya bean crop when endosulfan was sprayed on it. Acetylene reduction assay confirmed negative effect on soil nitrogenase.

Endosulfan was metabolized to endosulfan ether, lactone, hydroxyl ether and diol by hydrolytic pathway followed by *Anabaena sp.* ATCC 7210 grown for three days in medium supplemented with it (Shivaramaiah, 2010).

Endosulfan triggered toxicity of *Nostoc calcicola* and *Nostoc muscorum* revealed that insecticide suppressed nitrogenase activity, photosynthesis and photopigments when applied in range of 5-20 $\mu\text{g ml}^{-1}$. This study also indicated that *Nostoc muscorum* was more sensitive to endosulfan stress than *Nostoc calcicola* (Prasad et al., 2011).

Effect of endodhan on *Nostoc carneum* was investigated under *in vitro* conditions using insecticide alone and along with UV-B radiations (non ionizing) on protein, growth and photosynthetic pigments. Separate treatments with UV-B and endodhan had different inhibitory effects on above parameters. When endosulfan-treated samples were exposed to UV-B, however, an additive as well as synergetic impact was detected (Bhattacharyya et al., 2011).

Chlorophyll *a* fluorescence analysis was used to investigate the photosynthetic response of two rice field cyanobacterium *Anabaena* and *Nostoc sp.* to monocrotophos and endosulfan stress. Both species reacted to insecticide stress in dose and time dependent manner. These insecticides suppressed the growth by reducing production of photosynthetic pigments in the antenna complex, electron transport and PS- II. Monocrotophos was less inhibitory than endosulfan and *Anabaena sp.* was more susceptible to insecticide stress than *Nostoc sp.* (Nayak et al., 2012).

Four cyanobacterial species *Westiellopsis prolifica*, *Nostoc ellipsosporum*, *Tolypothrix tenuis* and *Scytonema simplex* was observed for changes when exposed to phorate, bagalol, mancozeb and thindan. The enzyme activity of glutamine synthetase, isocitrate lyase, isocitrate dehydrogenase and nitrogenase were suppressed along with growth at EC₅₀ values. *Westiellopsis prolifica* and *Tolypothrix tenuis* were most affected by phorate and thiodan. After 7 days of treatment, all pesticides induced a significant decrease in isocitrate lyase and isocitrate dehydrogenase activity (Debnath et al., 2012).

Kumar et al. (2011) used whole cell profiling technique for studying banding pattern of *Westiellopsis prolifica*, *Aulosira fertilissima* and *Anabaena fertilissima* exposed to 2, 4-D, pencycuron, endosulfan and tebuconazole which resulted in inhibition of protein content in dose dependent manner as confirmed by SDS PAGE analysis. Another study by Kumar et al. (2013) explained dissimilarities in 16S r DNA sequences, gap formation and mismatching of base pairs by studying native DNA structure of *Westiellopsis prolifica*, *Aulosira fertilissima* and *Anabaena fertilissima* exposed to endosulfan and tebuconazole.

2.3.4.4 Organophosphorus insecticides

2.3.4.4.1 Monocrotophos and Quinalphos

Synechococcus elongates, *Nostoc linckia* and *Phormidium tenue* metabolized quinalphos and monocrotophos when grown in different concentration ranging from 5-50 µg ml⁻¹ in liquid medium. TLC analysis of extracted residue confirmed formation of unidentified metabolites in both insecticides (Megharaj et al., 1987). Cyanobacterium *Aulosira fertilissima* utilized malathion, phosphamidon, monocrotophos, quinalphos, dichlorvos and phosphamidon as main source of phosphorus when grown in medium supplemented only with insecticide (Subramanian et al., 1994).

Rajendran et al. (2007) compared effect of bavistin, nimbicidin and monocrotophos on *Tolypothrix scytonemoides* and revealed that low dose of bavistin stimulated the growth while lower concentration of nimbicidin and monocrotophos increased respiratory consumption of oxygen but rate of photosynthetic evolution of oxygen was suppressed. Bavistin increased activity of nitrogenase but nimbicidin and monocrotophos decreased the nitrogenase and glutamine synthetase activity.

Monocrotophos treatment at $5\mu\text{g ml}^{-1}$ alone or with combination with exposure to UV-B irradiation ranging from 30-120 min to *Plectonema boryanum* drastically affected photosynthetic pigments, growth and photosynthesis (Prasad and Zeeshan, 2004). In cyanobacterium *Oscillatoria foreau* Fermi and *Anabaena ambigua* Rao, physiological parameters such as phenols, carbohydrates, protein, amino acids, ammonia were significantly affected upon treatment with $1-10\mu\text{g ml}^{-1}$ of insecticide divap 100 (Lakshmi and Annamalai, 2007).

2.3.4.4.2 Malathion and Phorate

Twenty-eight non-heterocystous filamentous cyanobacterial strains were tested against insecticide malathion along with dimecron and 2, 4 D revealed that *Oscillatoria acuminata* and *Lyngbya palmarum* accumulated more biomass while *Lyngbya spiralis*, *Lyngbya palmarum*, *Pseudoanabaena frigidum*, *Oscillatoria acuminata* and *Phormidium foveolarum* had shorter generation time. All the cyanobacterial species tolerated these pesticides as much higher concentration than recommended for paddy field (Tiwari et al., 2001).

Malathion stress in *Anabaena variabilis* was assessed by checking enzymatic antioxidant profile and osmolyte production when grown in medium supplemented with $25-100\mu\text{g ml}^{-1}$ of insecticide. The level of enzyme ascorbate peroxidase, superoxide dismutase and catalase were raised by test organism in range of 16-191% while important osmolytes such as mannitol, sucrose, trehalose, proline and glycogen were increased by 17-152% (Manikar et al., 2013).

Ibrahim et al. (2014) reported biodegradation of malathion when *Spirulina platensis*, *Nostoc muscorum* and *Anabaena oryzae* were grown in medium (phosphorus absent) containing insecticide resulted in utilization of phosphorus from insecticide.

Four cyanobacterial species were assessed for insecticide phorate in concentration range of 300-1000 $\mu\text{g ml}^{-1}$. These concentrations proved nontoxic for *Tolypothrix sp.*, *Westiellopsis sp.* and *Aulosira sp.* but toxic to *Nostoc sp.* even at 1 $\mu\text{g ml}^{-1}$ (Gangawane, 1979). Srinivasan and Ponnuswamy (1978), found that cyanobacterial population was increased when treated with 6 g/m² of ekalux.

2.3.4.4.3 Dimethoate and Triazophos

In nitrogen fixing cyanobacterium *Anabaena* PCC 7119, dimethoate treatment in concentration range of 20-300 $\mu\text{g ml}^{-1}$ significantly affected nitrogen fixation, growth, dark respiration and photosynthesis (Perona et al., 1991). Toxicity of dimethoate was studied on thylakoid pH gradient of *Synechocystis sp.* PCC 6803 resulted in instant increase of qN and decline in Fm at two different concentrations of 0.5 and 3 mM. This effect occurred due to inactivation of thylakoid ATPase due to insecticidal stress (Mohapatra et al., 1996). Mohapatra and Schiewer (1998) found that chlorfenvinphos and dimethoate at dose ranging between 0.1-0.5 mM affected the integrity of plasma membrane in different way. Treatment with Chlorfenvinphos and higher concentration of dimethoate resulted in leakage of membrane due to pH fluctuations while dimethoate treatment at low concentration inactivated H⁺-ATPase.

Chen et al. (2007) examined effect of dimethoate, bensulfuron-methyl and butachlor on growth and photosynthesis of *Nostoc* species. Drastic decline in growth was observed when 6-7 times more dose of these pesticides were applied than recommended. Inhibition of PSI and PSII activity was observed due to bensulfuron- methyl and butachlor while whole chain activity was affected due to dimethoate.

Four cyanobacterial species was assessed for toxic effect of insecticide trizophos. *Microcystis flos-aquae* was found to be most sensitive followed by *Anabaena flos-aquae*, *Microcystis aeruginosa* and *Chlorella pyrenoidosa* (least sensitive) (Ma et al., 2004).

2.3.4.4.4 Chlorpyrifos

A study was conducted to assess the effect of insecticide dursban 4E (chlorpyrifos) on *Synechococcus leopoliensis*. At recommended field application, no effect on growth of cyanobacteria was seen when grown in phosphate supplemented medium but in phosphate

limiting conditions the effects were more pronounced even at low concentration of $0.03 \mu\text{g ml}^{-1}$ (Van Donk et al., 1992). Carbofuran and chlorpyrifos biodegradation was studied by Jha and Mishra (2005) on cyanobacterium *Westiellopsis prolifica* and *Nostoc hatei* concluded that the later had more potential to degrade these insecticides.

Chlorpyrifos tolerance was checked on 180 isolated species of cyanobacterium in Punjab by Singh et al. (2007) concluded that ten isolates tolerated insecticide upto $20 \mu\text{g ml}^{-1}$, five isolates were able to grow upto $10 \mu\text{g ml}^{-1}$ and four isolates managed to show growth upto $5 \mu\text{g ml}^{-1}$. *Westiellopsis* TM was found to be most resistant (60% growth) followed by *Oscillatoria* KK7 (35% growth) and *Lyngbya* KKS (24% growth) in medium supplemented with $20 \mu\text{g ml}^{-1}$ of chlorpyrifos. The strains showing less than 20% growth were *Phormidium* JL-3, *Nostoc* GB14, *Phormidium* TMIS, *Synechocystis* TM and *Phormidium* GB10.

Chlorpyrifos induced oxidative stress was observed in terms of stimulated activities of glutathione-S-transferase, catalase, esterase, polyphenol oxidase and superoxide dismutase when *Phormidium valderianum* BDU 20041 was grown in $45 \mu\text{g ml}^{-1}$ of insecticide for 2 days (Palanisami et al., 2009). Chlorpyrifos induced inhibition in photosynthesis was observed in *Gloeocapsa crepidinum*, *Synechocystis aquatilis*, *Gloeocapsa sanguinea* and *Komvophoron minimum* having IC_{50} values 0.08, 0.074, 0.3 and $0.013 \mu\text{g ml}^{-1}$ respectively (Shoaib et al., 2012). Deviram et al. (2012) reported that insecticide dosage greater than $2.5 \mu\text{g ml}^{-1}$ adversely affected three cyanobacterial species *Oscillatoria*, *Nostoc* and *Anabaena* when grown in different concentrations of chlorpyrifos ranging between $0.5\text{--}4 \mu\text{g ml}^{-1}$. Thengodkar and Sivakami (2010) reported biodegradation of chlorpyrifos into trichloropyridinol by alkaline phosphatase enzyme of non-heterocystous filamentous cyanobacterium *Spirulina platensis*. Unicellular cyanobacterium *Synechocystis* sp. PUPCCC 64 was able to tolerate upto $15 \mu\text{g ml}^{-1}$ of insecticide and also reported to biodegrade chlorpyrifos into 3, 5, 6-trichloro-2- pyridinol as one of the metabolites, as confirmed by GC-MS chromatogram (Singh et al., 2011).

The effect of three organophosphorus insecticides methamidophos, terbufos and chlorpyrifos alone and in combination with each other was assessed on *Oscillatoria* sp. and *Chlorella* sp. by Tien and Chen (2012). EC_{50} value was in range of $0.33\text{--}7.99 \mu\text{g ml}^{-1}$ for *Oscillatoria* sp. and $1.29\text{--}41.16 \mu\text{g ml}^{-1}$ for *Chlorella* sp. upon treatment with single insecticide. *Chlorella* sp. was the more sensitive when mixture of pesticide treatment was applied. Antioxidant defence

system enzymes (SOD and CAT) were activated along with stimulated proline levels when *Chroococcus turgidus* NTMS12 was exposed to 6.9 and 12 $\mu\text{g ml}^{-1}$ of chlorpyrifos (Kumar et al., 2014).

Park and Lee (2010) reported that cytochrome P450 dependent monooxygenase of cyanobacterium *Anabaena* sp. PCC 7120 plays an important role in biodegradation of aldrin and chlorpyrifos methyl. Both aldrin and chlorpyrifos were converted to metabolite dieldrin and chlorpyrifos methyl oxon respectively.

2.3.4.4.5 Fenitrothion

Many cyanobacterial species were bleached when grown in presence of two different concentrations 5 and 10 $\mu\text{g ml}^{-1}$ of fenitrothion (Lal and Lal, 1998). Twelve different species of microalgae including cyanobacteria were severely affected at concentration of 10 $\mu\text{g ml}^{-1}$ of fenitrothion (Kent and Weinberger, 1991). Inhibition of growth of cyanobacteria was reported when treated with combination of pyridaphenthion and fenitrothion (Sabater and Carrasco, 2001a, b). When cyanobacterium *Microcystis aeruginosa* was treated with fenitrothion, inhibition in growth and photosynthesis was observed (Guanzon and Nakahara, 2002).

2.3.4.4.6 Parathion and Diazinon

Chlorella pyrenoidosa tolerated upto 100 $\mu\text{g ml}^{-1}$ of malathion and 72 $\mu\text{g ml}^{-1}$ of parathion when grown in media supplemented with insecticide (Christie, 1969). *Aulosira fertilissima* and *Cylindrospermum* sp. were able to grow well in diazinon concentrations of 400 and 300 $\mu\text{g ml}^{-1}$ respectively (Singh, 1973). Megharaj et al. (1994) reported biodegradation of 1000 $\mu\text{g ml}^{-1}$ of methyl parathion by cyanobacterial species (*Phormidium foveolarum*, *Nostoc muscorum*, *Oscillatoria animalis* and *Nostoc muscorum*) to undetectable levels within 20-30 days of treatment.

Methyl parathion showed fewer toxic effects than 2, 4-D when applied to cyanobacterium *Gloeocapsa*. The cyanobacterium showed stimulated growth with 1 $\mu\text{g ml}^{-1}$ while no notable effect was observed when grown in 5-100 $\mu\text{g ml}^{-1}$ of insecticide (Tozum-Calgan and Sivaci-Guner, 1993).

Filamentous nitrogen fixer *Cylindrospermum* sp. was evaluated for toxicity of insecticide methyl parathion revealed that low dose ($0.5 \mu\text{g ml}^{-1}$) of insecticide proved stimulatory while high dose ($8 \mu\text{g ml}^{-1}$) resulted in formation of akinetes under *in vitro* conditions. Under field conditions, LC_{50} and LC_{100} values for insecticide were 13.6 and $30 \mu\text{g ml}^{-1}$ respectively (Panigrahi et al., 2003).

Under photoautotrophic aerobic conditions, *Anabaena* sp. PCC 7120 metabolized methyl parathion into o, o-dimethyl-o, and p- aminophenyl thiophosphate (Barton et al., 2004) Fioravante et al. (2010) reported that Cyanobacterium *Microcystis novacekii* removed the methyl parathion from the medium by mineralizing it without forming any degradation product.

Two cyanobacterial isolates *Oscillatoria tenue* and *Anabaena cylindrica* was grown in $1\text{-}10 \mu\text{g ml}^{-1}$ of insecticide diazinon for 28 days revealed that higher dose of insecticide ranging from $9\text{-}10 \mu\text{g ml}^{-1}$ deleteriously affected growth, nitrogen, amino acids, protein and carbohydrate content in heterocystous forms than non heterocystous forms (Ghadai et al., 2010a).

2.3.4.4.7 Trichlorfon and Fenamiphos

The trichlorfon drastically inhibited nitrate reductase activity and nitrogen source uptake (nitrate and ammonium) in *Anabaena* PCC 7119 (Marco and Orus, 1993). Limited uptake of nitrate along decreased phycobilins and chlorophyll *a* was observed in *Plectonema calothricoides*, *Gloeotheca*, *Chlorogloeopsis* and *Nostoc species*. Trichlorofon, along with dimethoate, phoxim and glyphosate inhibited chlorophyll *a* fluorescence of *Microcystis wesenbergii* after 4 days of treatment at different doses of 6.84, 13.48, 148.52, 382.34, $\mu\text{mol l}^{-1}$ respectively (Sun et al., 2013).

Megharaj et al. (1999b) studied effect of fenamiphos on cyanobacteria and its enzymatic activities such as arylsulphatase, β -glycosidase and dehydrogenase in soil. Higher concentrations of fenamiphos ranging from 5-10 kg/ha had very little effect on cyanobacteria.

Five different cyanobacterial strains *Nostoc muscorum*, *Anabaena sp.* *Nostoc* MM1, MM2, MM3 showed potential to metabolize fenamiphos into its primary metabolite fenamiphos sulfoxide phenol (Caceres et al., 2008).

The cyanobacterial and other algal species on which insecticides effect has been studied are listed in Table 2.8:

Table 2.8: Cyanobacterial and other algal species on which insecticides effect has been studied

S.No.	Insecticide used	Cyanobacterial species	Reference
1.	Carbaryl, DDT, Lindane	<i>Monochrysis lutheri</i> , <i>Dunaliella euchlora</i> , <i>Chlorella</i> sp., <i>Protococcus</i> sp., <i>Phaeodactylum tricornutum</i>	Ukeles, 1962
2.	Carbaryl	<i>Chlorella pyrenoidosa</i> , <i>Nostoc muscorum</i> , <i>Cylindrospermum</i> , <i>Microcystis aeruginosa</i> , <i>Oscillatoria</i> sp., <i>Anabaena inaequalis</i> , <i>Anabaena Flos-aquae</i>	Christie, 1969
3.	Endrin, Lindane, Benzene Hexachloride	<i>Cylindrospermum</i> sp., <i>Aulosira fertilissima</i> , <i>Plectonema boryanum</i>	Singh, 1973
4.	P, P9-DDT	<i>Selenastrum capicornutum</i> , <i>Chlorella</i> sp., <i>Anabaena</i> sp., <i>Cylindrospermum</i> sp., <i>Aulosira fertilissima</i>	Singh, 1973
5.	Dieldrin, Eldrin	Freshwater Algae	Clegg and Koevenig, 1974
6.	Diazinon, Methoxychlor, Carbyl	Planktonic Algae	Butler et al., 1975
7.	Methoxychlor, Mirex	<i>Chlorella pyrenoidosa</i>	Kricher et al., 1975
8.	DDT	<i>Selenastrum capricornutum</i>	Lee et al., 1976
9.	Fenitrothion	<i>Chlorella vulgaris</i> , <i>Chlorococcum hypnosporum</i> , <i>Oscillatoria lutea</i> , <i>Tribonema</i> , <i>Stigeoclonium tenue</i> , <i>Vaucheria geminata</i>	Torres and Flaherty, 1976
10.	Chlorpyrifos	<i>Minutocellus polymorphus</i>	Walsh et al., 1988
11.	Dimethoate	<i>Scenedesmus Incrassatulus</i>	Jampani and Kumari, 1988
12.	Endosulfan	<i>Anabaena</i> sp., <i>Aulosira fertilissima</i>	Tandon et al., 1988.
13.	Endosulfan	<i>Westiellopsis prolifica</i>	Adhikary, 1989
14.	Dimethoate,	<i>Chlorella vulgaris</i> , <i>Anabaena doliolum</i>	Mohapatra and

S.No.	Insecticide used	Cyanobacterial species	Reference
	Endosulfan		Mohanty,1992
15.	Carbofuran	<i>Scenedesmus quadricauda</i> , <i>Selenastrum cupricornutum</i> , <i>Nitzschia</i> sp., <i>Cyclotella meneghiana</i> , <i>Microcystis aeruginosa</i> , <i>Oscillatoria</i> sp., <i>Pseudoanabaena</i> sp., <i>Anabaena inaequalis</i> , <i>Aphanizomenon flos-aquae</i>	Peterson et al., 1994
16.	Lindane, Parathion	<i>Chlorella fusca</i>	Faust et al., 1994
17.	Carbyl	<i>Scenedesmus Quadricauda</i> , <i>Selenastrum Capricornutum</i> , <i>Anabaena</i> sp.	Peterson et al.,1994
18.	Trichlorfon	<i>Scenedesmus bijuga</i>	Fathi, 2003
19.	Carbyl, Carbofuran	<i>Anabaena flos-aquae</i> , <i>Microcystis flos-aquae</i> , <i>M.aeruginosa</i> , <i>Selenastrum capricornutum</i> , <i>Scenedesmus quadricauda</i> , <i>Scenedesmus</i> <i>Obliquus</i> , <i>Chlorella vulgaris</i> , <i>Chlorella Pyrenoidosa</i>	Ma et al., 2006
20.	Chlorpyrifos	<i>Phormidium valderianum</i>	Palanisami, 2009
21.	Bagalol, Mancozeb	<i>Nostoc ellipsosporum</i> , <i>Scytonema simplex</i> , <i>Tolypothrix tenuis</i> , and	Debnath et al., 2012
22.	Thiodan Phorate	<i>Westiellopsis prolifica</i>	
22.	Carbaryl	<i>Calothrix brevissima</i>	Habib et al., 2011
23.	Pentachlorophenol	<i>Microcystis aeruginosa</i> , <i>Chlorella vulgaris</i>	De Moaris et al., 2014
24.	Chlorpyrifos	<i>Chroococcus turgidus</i>	Kumar et al., 2014a
25.	Cyhalothrin	<i>Calothrix</i>	Gupta and Baruah, 2015
26.	Cartap hydrochloride	<i>Leptolyngbya foveolarum</i>	Singh et al., 2014
27.	Profenofos	<i>Anabaena</i> PCC 7120	Tiwari et al., 2016
28.	Malathion	<i>Anabaena sphaerica</i>	Chakraborty et al., 2017

S.No.	Insecticide used	Cyanobacterial species	Reference
29.	Methyl parathion	<i>Fischerella sp.</i>	Tiwari et al., 2018
30.	Cypermethrin	<i>Anabaena</i> PCC 7120 and <i>Nostoc muscorum</i> ATCC 27893	Tiwari and Prasad, 2020
31.	Deltamethrin	<i>Calothrix sp. (gueco 1002)</i>	Gupta and Baruah, 2020
32.	Pyrethroid	<i>Chroococcidiopsis sp. and Microcystis sp.</i>	Bano et al., 2021
33.	Pyridaphenthion	<i>Anabaena laxa and Nostoc muscorum</i>	Hamed et al., 2021

2.4 Nanoformulations of Pesticides

Nanotechnology involves the synthesis of materials with dimensions in the range of nanometers (10^{-9} m) by manipulating atoms and molecules (Kaur et al., 2021). Concoction that includes elements in the nm size range and claims novel characteristics linked with pesticides in these small size ranges is referred to as nanopesticide. Nanopesticides are a diverse group of products that cannot be placed together into a single category. These nanotechnology-based attempts include polymeric micelles, vesicular delivery systems (liposomes), dendrimers, hydrogel, nanoemulsion, nanohybrid scaffolds, nanostructured lipid carriers (NLCs), PLGA NPs, electrospun nanofibers, and polymeric nanoparticles (NPs) (Rawtani et al., 2018) (Fig. 2.7).

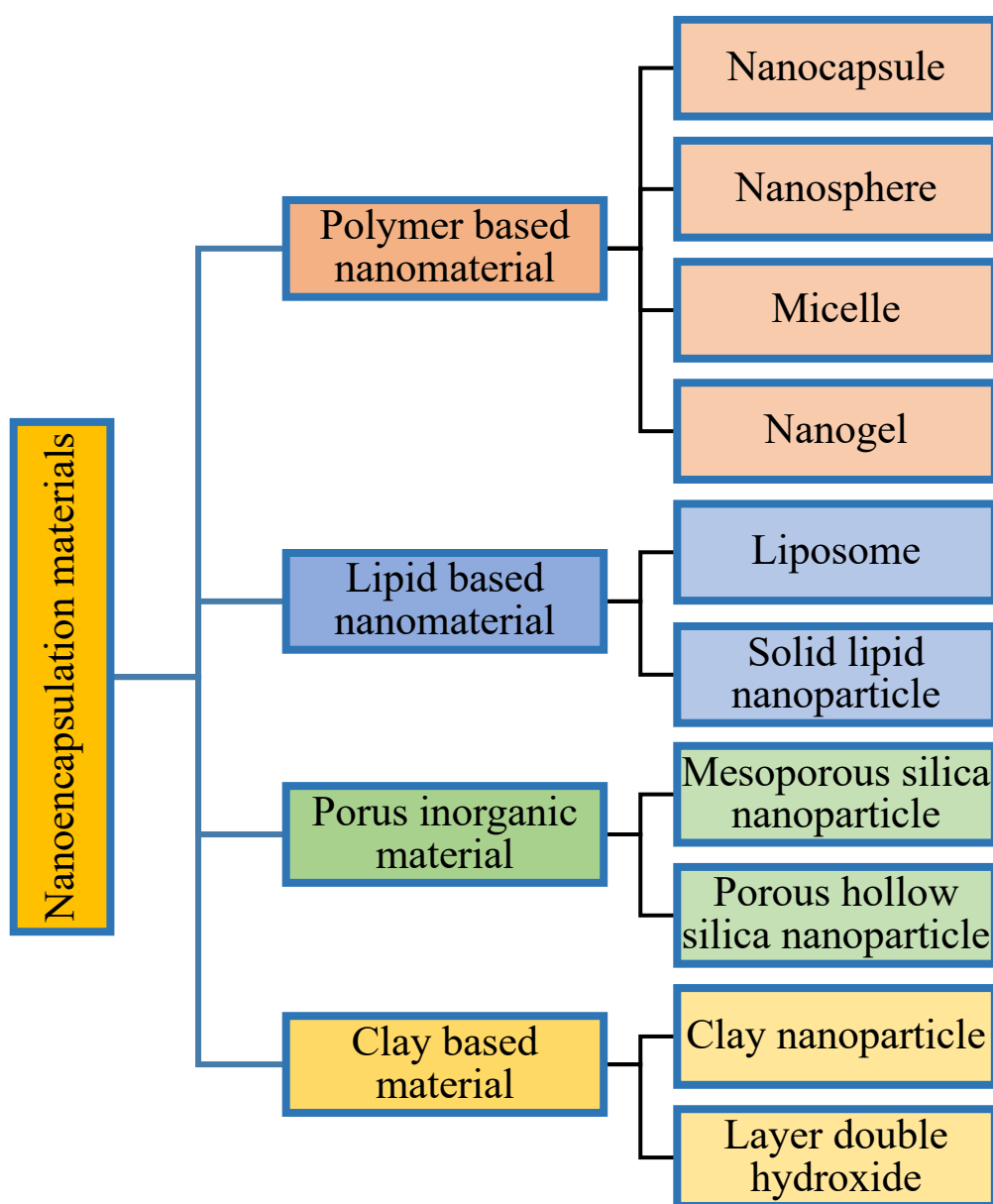


Fig. 2.7: Encapsulation material for pesticide delivery

The summary of improvements made in the delivery of insecticides by adopting nanotechnology-based delivery system is presented in Fig. 2.8.

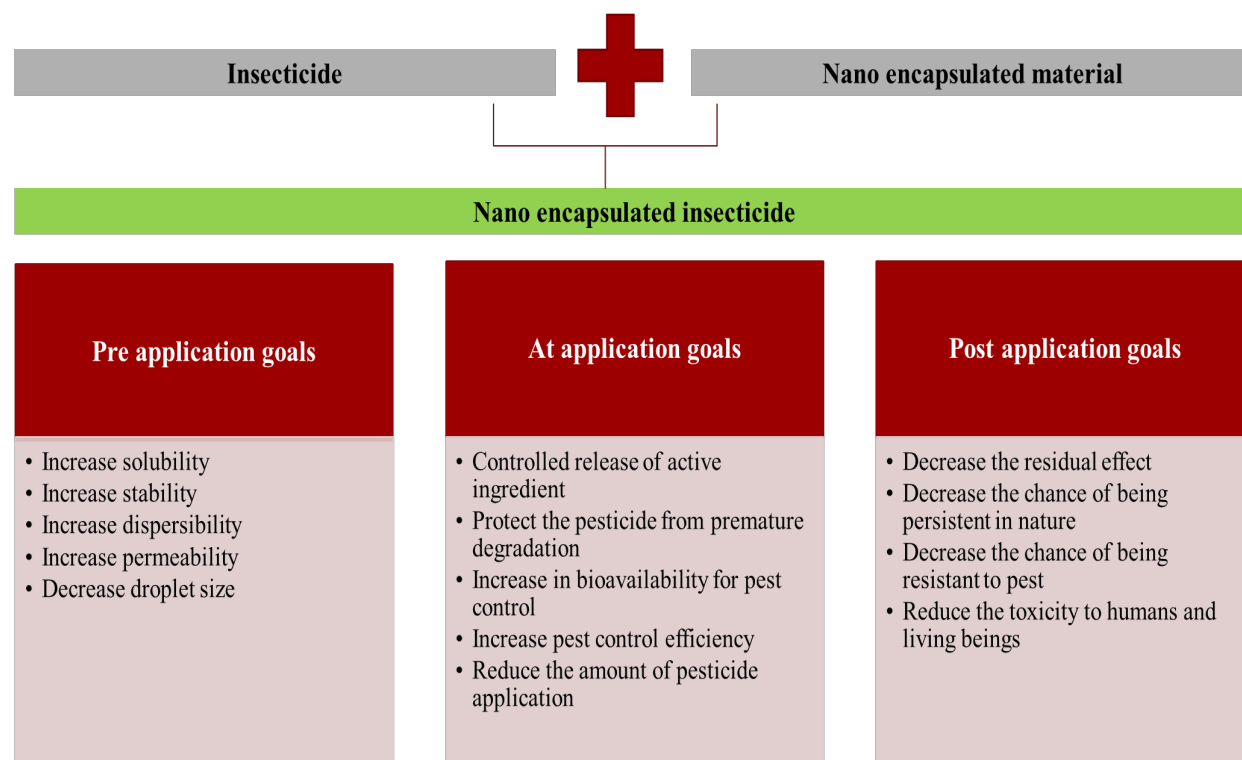


Fig. 2.8: Schematic diagram exploring the application goals of the nanotechnology based advanced delivery systems in improving the potential of insecticide delivery

Nanopesticides can be made up of inorganic (metal oxides) and organic materials (polymers) and in various forms (particles and micelles). The primary aim of nanoformulation is to release active ingredient in controlled manner to prevent its degradation and to improve solubility of poorly soluble active ingredient. Natural polymer are used in variety of ways to synthesise controlled release (CR) formulations. When employed alone, a natural polymer isn't very effective as a CR device but when it is linked to any cross-linkers, sorbent, grafted with another polymer its effectiveness and stability increases. Natural carbohydrate polymers, such as chitosan (CS), alginate (ALG), pectin etc. are brought into light as these are cost-effective polymers and are biocompatible, biodegradable (Mukhopadhyay., 2015). Chitosan (β -1, 4-linked polysaccharide) is a cationic polymer produced in bulk from chitin (crustaceans shells) as a by-product (Fig. 2.9) (Agnihotri et al., 2012; Gholamali et al., 2020). Because of its extraordinary properties, such as gel forming capability, low toxicity, biodegradability, biocompatibility and eco-friendliness (Agnihotri et al., 2012; Umma et al., 2019) made it appropriate choice for synthesizing nanoformulations. Alginate is a naturally

occurring anionic biopolymer linked by β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues (Fig. 2.9), is non-immunogenic, non-toxic, biocompatible and biodegradable (Kloareg and Quatrano, 1988; Smidsrod and Draget, 1996; Smidsrod et al., 1966).

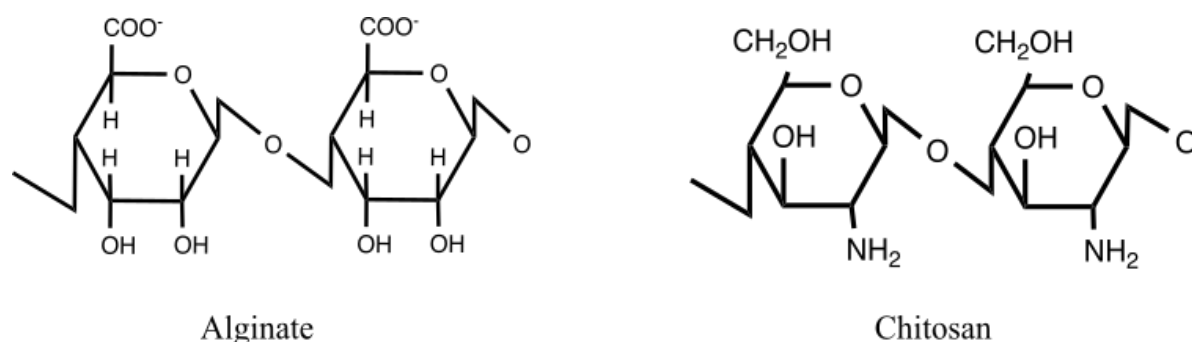


Fig. 2.9: Structure of chitosan and alginate

Through the gelation phenomenon, divalent cations such as Ba^{2+} and Ca^{2+} can rapidly form egg-box complexes with G block to produce alginate gel (Tavakoli et al., 2019). Gelling qualities of alginate and its simple processing made them most biosynthesized polymers giving benefits in various sectors such as pharmaceutical, food industry and agriculture (Salleh et al., 2019; Pawar and Edgar, 2012; Ismail et al., 2016; Lee and Mooney, 2012). Alginate is considered an environmentally friendly polymer because it can be recycled and degraded.

Alginate's physicochemical properties, such as viscosity, thermo stability, sol-gel transformation, pH-responsiveness, and drug release, can help researchers better understand its potential applications (Loquercio et al., 2015). Different methods such as microemulsion gelation, emulsification solvent diffusion, ionotropic gelation, reverse micellar, polyelectrolyte complexation can be employed for the formation of nanoformulations using natural polymer which are described in Fig. 2.10.

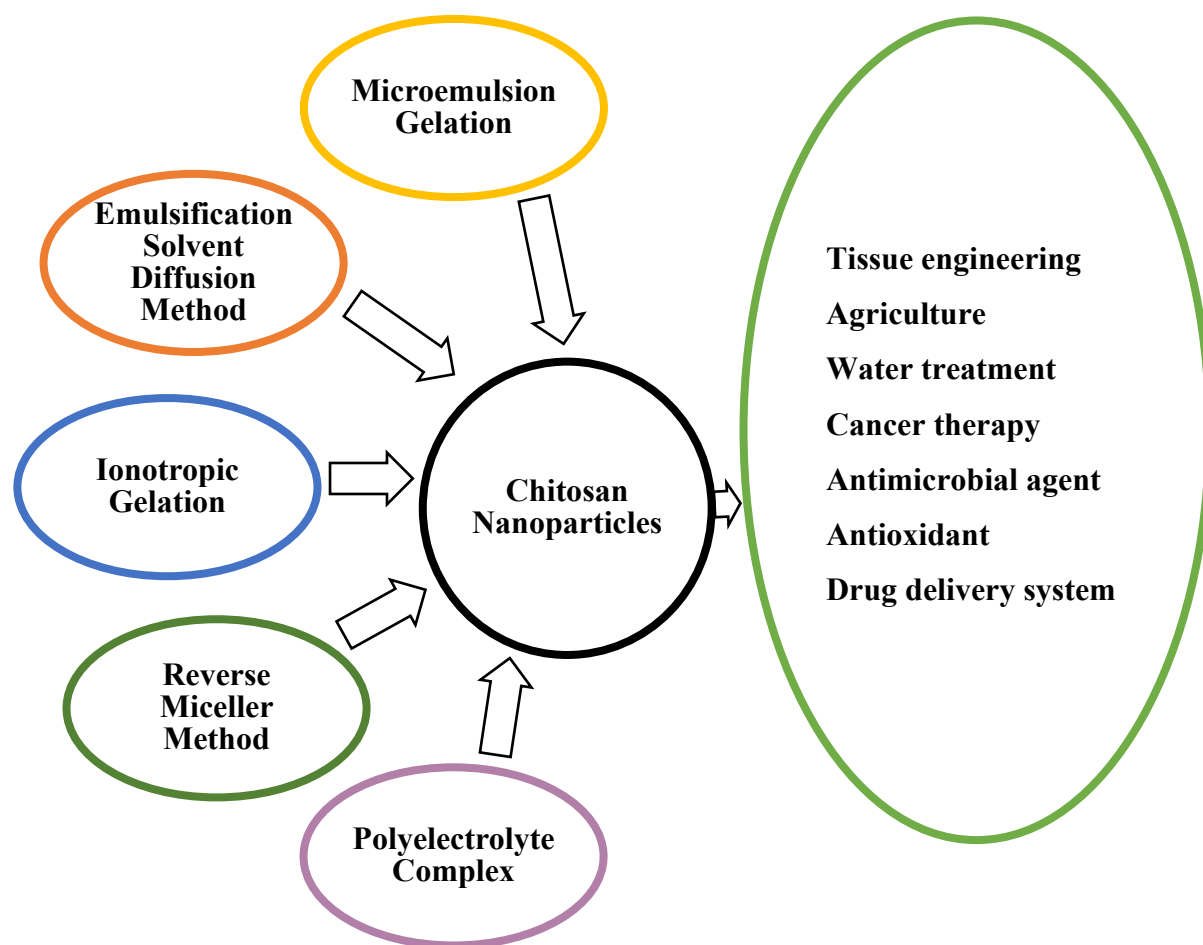


Fig. 2.10: Different methods employed in nanoformulation synthesis

Several authors have conducted experiments on the controlled release of pesticides by utilizing alginate. To determine the controlled release of atrazine (Fernandez et al., 2004), metribuzine, chloridazon (Flores et al., 2007), carbofuran (Fernandez et al., 2000), diuron ((Fernandez et al., 1999), imidacloprid, cyromazine and isoproturon (Garrido et al., 2006), a standard herbicide alginate water formulation was modified by adding different sorbents, such as bentonite and activated carbon. All these sorbent-modified formulations had a high encapsulation efficiency (EE) in range of 83–98%, and the release kinetics of pesticides from formulation was controlled by diffusion.

In these formulations, pesticide leaching was slowed down to 50 and 75 % when natural and acid treated bentonite was used (Fernandez et al., 2000). The solubility of pesticides in water also plays an important role in their release. Water-soluble insecticides were released very fast from formulation and have low encapsulation efficiency than hydrophobic insecticides

(Flores et al., 2007). The addition of lignin to alginate-sorbent formulations of cyromazine, isoproturon, chloridazon, metribuzin and imidacloprid increased their release in water (Fernandez et al., 2010). Cypermethrin nanoformulation was synthesized by crosslinking of alginate-gelatin beads with calcium chloride. Loading concentration of insecticide had effect on its release rate from the matrix (Roy et al., 2009a). Controlled release formulation of thiram was prepared by crosslinking of barium chloride and aluminium chloride with starch alginate beads separately. Barium chloride linked beads showed more efficient crosslinking and release behavior than aluminium chloride linked beads (Singh et al., 2009a).

Casanova et al. (2005) used a sequence of fatty acids (C10–C18) and a surfactant to produce a nicotine carboxylate nanoemulsion. The size of particle was 100nm, with monomodal distribution of size in the oil-in-water nanoemulsion. The LT_{50} was used to test the bioactivity of the insecticidal formulations. As the size of the fatty acids increased, the encapsulation efficiency and bioactivity of nanoemulsion declined.

Nano-hexaconazole and nanosulfur nanoformulation was synthesised and characterized by SEM, TEM, and FTIR showed nanoparticle size less than 100nm. Both the formulations were very effective in controlling insects. Nano hexaconazole was five times more powerful in controlling pathogens and nanosulfur was more effective (10 times) than its commercial water soluble formulation (Gopal et al., 2011).

Chitosan alginate nanoparticle incorporated with paraquat was synthesised and characterization by FTIR, TEM and DSC which confirmed its loading into nanoparticles. The size of nanoparticle was 635 ± 12 nm with polydispersity of 0.518, zeta potential of -22.8 ± 2.3 mV and encapsulation efficiency of 74.2%. Release profile of encapsulated paraquat confirmed that it reduced negative impact on soil microorganisms and released in controlled manner (Mariana et al. 2011).

Nanocomposite Functional hybrid of 4-chlorophenoxy acetate (4-CPA) and 2,4Dichlorophenoxy acetate (2, 4-D) was synthesised by intercalation of these anions by layer double hydroxide (LDH) type of Zn, Al synthesised by co precipitation. Characterization techniques (X-Ray Diffractions (XRD), Fourier Transformation Infrared (FTIR), thermal analysis. (TG- DTG) and gas chromatography coupled with mass

spectroscopy (GC-MS) confirmed the presence of a 2, 4-D and 4-CPA combination (Abbas et al., 2011).

Controlled release nanoformulation of imidacloprid [1-(6 chloro-3-pyridinyl methyl)-N-nitro imidazolidin-2-ylideneamine] was synthesised by using nano-micellar aggregates of poly (ethylene glycols) and various aliphatic diacids using encapsulation. These amphiphilic polymers' high solubilization power and low critical micelle concentration (CMC) improved formulation efficacy. DLS, FTIR, and TEM confirmed its loading, Release kinetics confirmed that nanoformulation was better than commercial formulation and efficient for pest management (Adak et al., 2012).

Atrazine nanocapsules were prepared by using poly (ε-caprolactone) and coated by different doses of chitosan. 64-84% encapsulation efficiency was achieved in nanoformulation. When the nanocapsules were coated, the herbicide's release kinetics profile changed, and the release mechanism was summarized as a combination of diffusion and relaxation of the polymeric chains. The concentration of chitosan used in the synthesis of nanoformulation had an impact on the formulations' stability (Grillo et al., 2014b).

Norhayati et al. (2014) used a co-precipitation and direct reaction approach to synthesise two phenoxy herbicide nanocomposite of cloprop-zinc-layered hydroxide and cloprop-layered double hydroxide nanocomposite. Both nanocomposite were completely intercalated between the interlayer's metal hydroxide as confirmed by elemental studies and FTIR. Release kinetic studies of both kind of nanoformulations confirmed their controlled release into the system. Release of cloprop from nanoformulation followed pseudo second order kinetics for cloprop-layered double hydroxide nanocomposite however for cloprop-zinc-layered hydroxide nanocomposite the release was governed by parabolic diffusion. The results confirmed that both type of composites have potential for controlled release of cloprop.

Sodium alginate nanoparticles are valuable tool to for pesticide delivery as they possess very low toxicity, simple and cheap synthesis methods, biodegradability and better stability. Imidacloprid nanoparticles were synthesised by using sodium alginate having average nanoparticle size ranging between 50-100nm with encapsulation efficiency of 98.66%. Efficacy trials on okra confirmed that encapsulated insecticide is more effective than commercial formulation and least toxic to non-target microorganisms (Kumar et al., 2014)

Maruyama et al. (2016) synthesised two types for nanoformulations of imazapyr and imazapic using chitosan-tripolyphosphate and chitosan- alginate for their controlled release. Nanoparticles were evaluated for their encapsulation efficiency, toxicity and release kinetics. The average particle size of chitosan tripolyphosphate nanoparticles loaded with imazapyr and imazapic was 478.6 nm and zeta potential of + 26 mV having encapsulation efficiency ranging between 50-70%. The release of imazapic was by diffusion following Fick's law, described by the Higuchi model and the release of imazapyr obeyed the Korsmeyer-Peppas mathematical model and indicated release by particle dissolution. In case of chitosan alginate nanoparticles, average particle size with imazapyr and imazapic was 377.7 nm having zeta potential of -30 mV and encapsulation efficiency in range of 62-72%. Release kinetics revealed that imazapic was released via diffusion following Fick's law, but imazapyr was released via Case II transport, indicating a different method of interaction between the imazapyr and the nanospheres. Cytotoxicity studies confirmed that they are less hazardous than plain pesticide.

Nanoformulation of insecticide nicotine hydrochloride (NCT) was synthesized by using chitosan and sodium tripolyphosphate to decrease its negative effects on humans and to improve its stability. The nanoformulation was stable for 45 days, have encapsulation efficiency of 55% and was thermally stable. Physiochemical characterization of nanoformulation confirmed their ionic crosslinking (Yang et al., 2018).

The common polymers (synthetic and natural ones) used in CRFs for insecticides application are listed in Table 2.9.

Table 2.9: Polymer used in nanoparticle production

S. No	Polymer	Active compound	Nanomaterial	Reference
1.	Vinyl ethylene and vinyl acetate	Pheromones	Resin	Wright, 1997
2.	Lignin	Aldicarb	Gel	Kok et al., 1999
3.	Alginate-glutaraldehyde	Neem Seed Oil	Capsule	Kulkarni et al., 1999
4.	Glyceryl ester of fatty acids	Carbaryl	Spheres	Quaglia et al., 2001
5.	Polyvinylchloride	Chlorpyrifos	Particle	Liu et al., 2002
6.	Methyl methacrylate and methacrylic acid with and without 2-hydroxy ethyl methacrylate crosslinkage	Cypermethrin	Gel	Rudzinski et al., 2003
7.	Carboxymethylcellulose	Carbaryl	Capsule	Isiklan, 2004
8.	Acrylic acid-Buacrylate	Itraconazole	Capsule	Goldshtein et al., 2005
9.	Poly(butyl acrylate), Polyvinyl alcohol, Polyvinylpyrrolidone	Bifenthrin	Capsule	Liu et al., 2008
10.	Polyethylene glycol	Garlic Essential Oil	Capsule	Yang et al., 2009a
11.	Chitosan alginate	Cypermethrin	Suspension	Roy et al., 2009a
12.	Polyethylene glycol-dimethyl esters	Carbofuran	Micelle	Shakil et al., 2010
13.	Polyethylene	Piperonyl Butoxide and Deltamethrin	Capsule	Frandsen et al., 2010
14.	N-(octadecanol-1-glycidyl ether)-O-sulfate chitosan-octadecanol glycidyl ether	Rotenone	Micelle	Lao et al., 2010
15.	Polyvinylpyrrolidone Anionic surfactants (naphthalene sulfonate condensate sodium salt and sodium dodecyl sulfate)	Novaluron	Powder	Elek et al., 2010
16.	Chitosan-poly(lactide)	Imidacloprid	Particle	Li et al., 2011
17.	Poly (methyl methacrylate) - poly (ethylene glycol)	Carbofuran	Suspension	Chin et al., 2011

S. No	Polymer	Active compound	Nanomaterial	Reference
18.	Lignin	Imidacloprid, Cyromazine	Granules	Fernandez-Perez et al., 2011
19.	Zn/al layered double hydroxide cloprop-LDH)	2,4 D	Powder	Abbas et al., 2011
20.	Sodium alginate and chitosan	Paraquat	Suspension	Mariana et al., 2011
21.	Chitosan	Etofenprox	Capsule	Hwang et al., 2011
22.	Alginate-bentonite	Imidacloprid	Clay	Fernandez-Perez et al., 2011
23.	Glycol-ethylcellulose	Cyromazine		
24.	Polyamide	Pheromones	Fiber	Hellmann et al., 2011
25.	Zn/al layered double hydroxide (cloprop-LDH) and Zn layered double hydroxide (cloprop-ZLH)	Cloprop	Powder	Norhayati et al., 2014
26.	Sodium alginate	imidacloprid	powder	Kumar et al., 2014
27.	Carbon nanotubes	Mancozeb, Zineb	Suspension	Sarlak et al., 2014
28.	Poly(e-caprolactone) and chitosan	Atrazine	Capsule	Grillo et al., 2014b
29.	Chitosan and alginate	Acetamiprid	Capsule	Kumar et al., 2015
30.	Chitosan and tripolyphosphate	Paraquat	suspension	Grillo et al., 2015
31.	Chitosan and tripolyphosphate	Imazapic and imazapyr	suspension	Maruyama et al., 2016
32.	Alginate and chitosan	Imazapic and imazapyr	Sphere	Maruyama et al., 2016
33.	Chitosan	Hexaconazole	Suspension	Chauhan et al., 2017

Conclusion

Extensive application of cartap could lead to accumulation of its residues at dangerous levels which may affect the non-target microorganism including cyanobacteria. The effect of cartap on stress response by heterocystous cyanobacteria such as *Anabaena variabilis* has till date, not been substantiated. There are several reports of applications of polymer nanoformulations in human medicine but very less is reported on controlled release of pesticides using polymer nanocapsules. Encapsulation of agrochemicals in nanoparticle becomes an eco-friendly approach towards their effective utilization in agriculture. Previous literature revealed that such studies related to cartap hydrochloride have not been carried out. Therefore, present research was undertaken to study the effect of cartap hydrochloride on stress response and different physiological activities of *Anabaena variabilis* and to synthesize nanoformulation of cartap hydrochloride and to study its release kinetics with the following objectives:

1. Effect of cartap hydrochloride on growth and physiological parameters of heterocystous cyanobacteria
2. Nanoformulation of cartap hydrochloride and its characterization
3. Evaluation of release kinetics of nano-cartap hydrochloride

3.1 Material

3.1.1 Microorganism and Culture Conditions

The cyanobacterium *Anabaena variabilis* strain ARM 441, was procured from the National Centre for Conservation and Utilization of Blue green algae, Division of microbiology, IARI, New Delhi, India. The cyanobacterium is filamentous and heterocystous belonging to order Nostocales of class Cyanophyceae. It was grown in BG-11 medium (-N) (Stanier, 1977). Cultures were maintained in a room at $28\pm 2^{\circ}\text{C}$ under discontinuous illumination 16h: 8h light dark cycle at 2500-3000 lux light intensity. Exponentially growing cultures were used in our study and each experiment was repeated thrice.

3.1.2 Chemicals and Glassware

Cartap hydrochloride (98.9%) technical grade was procured from Crop Chemical House, Kot-Kapura, Punjab, India. Rest of the chemicals used was obtained from Sigma Aldrich, SD fine chemicals and Merck. Glassware and plasticware used in the study was procured from Tarsons, India. All the glassware and solutions were steam sterilized in autoclave for 15-20 min at steam pressure of 15 lbs inch⁻².

3.1.3 Propagation of cultures

The cyanobacterial cultures were grown in the BG 11 medium at a temperature of $28\pm 2^{\circ}\text{C}$ and after 6-7 days transferred to fresh medium to keep them in exponential phase. Fluorescent tubes having intensity of $44.5\ \mu\text{ moles of photons m}^{-2}\text{ s}^{-1}$ (μE) were used for illumination of cultures and shaking was done twice daily to keep them in homogenous state.

3.2 Growth of *Anabaena variabilis* ARM 441 in cartap exposure

Growth was checked in terms of dry biomass. The tolerance limit of *Anabaena* sp. against insecticide was measured by growing it in graded concentrations of cartap ranging between $5\text{--}100\ \mu\text{g ml}^{-1}$. These concentrations were prepared from the stock solution of technical grade cartap hydrochloride (98.9%). Exponentially growing stock cultures were inoculated in cartap containing media to get the initial absorbance of 0.01 at 720 nm. At regular intervals of 3 d, extending up to 21 d, 10 ml samples were withdrawn, filtered and oven dried (60°C) for overnight and dry biomass was expressed as mg ml^{-1} . The Specific growth rate was estimated

as biomass yield (Singh et al., 2012a). Specific growth rate (μ) was defined as the derivative of population with respect to time over population using the following equation.

$$\frac{1}{N \left(\frac{dN}{dt} \right)} = d \frac{\ln N}{dt}$$

Where N and t stands for population and time, respectively.

3.3 Protein and Carbohydrate estimation

Protein estimation was done by method given by Lowery et al. (1951). Known amount of cyanobacterial suspension was taken, centrifuged and washed with distilled water. The pellet was retained and NaOH (1N) was added to it to digest cells and place in boiling water bath for 10 min to get protein extract. The resultant mixture was centrifuged and supernatant further used for protein estimation. To 1 ml of extract, solution C (5 ml) was added. After 10 min of incubation at room temperature, solution D (0.5 ml) was added to reaction cocktail and again incubated for 20min. The absorbance of coloured product (blue) was recorded at 660 nm, considering bovine serum albumen as standard.

Carbohydrate content was measured by using anthrone method (Manikar et al., 2013). Known amount of cyanobacterial suspension was centrifuged and pellet was washed twice with distilled water. 1.25 ml of distilled water was added to cyanobacterial biomass along with 4 ml of anthrone reagent and incubated in boiling water bath for 10 min. Absorbance of samples were taken at 620 nm considering glucose as standard.

3.4 Photosynthetic pigments

Cyanobacterial culture was centrifuged and washed with double distilled water. Equal volume of acetone (80%) was added to the cell pellet and kept at room temperature for 12 h to extract acetone soluble photosynthetic pigments. The extracts were centrifuged and attained the same volume of supernatant by adding sufficient amount of acetone and absorbance was checked at 660, 645 and 450 nm. The amount of chlorophyll *a* (Chl *a*) was calculated by using method as described by Holm (1954) and total carotenoids (Car) were quantified as per Myers and Kratz (1955). Phycobiliproteins (PBS) that constitutes phycocyanin, phycoerythrin and allophycocyanin. PBS were extracted by freeze thawing method in 50 mM phosphate buffer (pH 7.0) (Singh et al., 2013). The cyanobacterial suspension was centrifuged (5000 rpm), washed thrice with distilled water and pellet was

resuspended in phosphate buffer containing lysozyme (1 g l⁻¹) and incubated in water bath for 1h at 37°C with continuous stirring. The cyanobacterial suspension was then subjected to 10–12 freeze-thawing cycles until all the pigments were released from the cells. After centrifugation at 5000 rpm, the absorbance of the supernatant was recorded at 565, 615 and 652 nm.

3.5 Absorption spectra

Absorption spectrum of insecticide treated and control cultures were recorded in absorption range of 400-700 nm by using UV Visible spectrophotometer (Thomas and Nagaraja, 1973). Double diffusion technique was used to record the spectrum in which sample and reference were placed on same height so that their light beams scatter to same extent. 3 ml of algal suspension (5µg of Chl ml⁻¹) was taken to record spectra after 21 days of incubation.

3.6 Heterocyst frequency

Heterocyst frequency was observed microscopically by fixing material in Lugol's iodine solution (5g iodine in 100 ml of 10% potassium iodide solution). Heterocyst was observed under 100X objective (oil immersion) and frequency was calculated as follows:

$$\text{Heterocyst frequency (\%)} = \frac{\text{Total no. of heterocyst}}{\text{Total no. of cells}} * 100$$

3.7 Photosynthesis and respiration

Photosynthesis and dark respiration was measured according to Singh et al. (2014) in terms of evolution and consumption of oxygen in light and dark respectively. Cyanobacterial culture (10 µg Chl *a* ml⁻¹) was treated for different time interval of 24 and 48 hr with different insecticide concentration. Known amount of culture was taken and centrifuge to get thick suspension. The pellet was resuspended in very small amount of BG11 medium in a vessel fitted with oxygen analyzer (HI 9147). After incubation under light source (225 µe on the vessel surface) for 10 min, the increase in amount of oxygen was checked. In the similar way, for determining the rate of dark respiration, the cyanobacterial suspension was placed in reaction vessel fitted with oxygen analyzer was incubated under dark conditions for ten minutes to check decrease in oxygen level. The amount of increase and decrease in oxygen level with respect to different concentrations of insecticide was calculated as nmol O₂ evolved/ consumed µg⁻¹ Chl *a* min⁻¹.

3.8 Enzymatic antioxidants

3.8.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was determined by the method of Beauchamp and Fridovich (1971) in terms of inhibition of reduction of dye nitroblue tetrazolium chloride (NBT). Cyanobacterial suspension was centrifuged, washed with distilled water and resuspended in phosphate buffer (100 mM, pH 7.8). The sample was sonicated (10min) (sonics Vibra cell VC 750) and centrifuged and supernatant was used as an enzyme extract. To this enzyme extract, methionine (3%), NBT (0.014%), EDTA (2mM), Na₂CO₃ (23.4%), sodium phosphate buffer (100 mM, pH 7.8) and riboflavin (0.015%) were added. Test tubes were incubated under fluorescent light for 5 min and then transferred to dark condition to terminate the reaction. The absorbance of reaction mixture was taken at 560 nm. One unit of SOD activity is defined as the amount of enzyme required to inhibit the reduction of NBT in light by 50%.

3.8.2 Peroxidase (POD)

Peroxidase (POD) activity was determined according to the method of Gahagen et al. (1968). The reaction mixture contained phosphate buffer (100 mM, pH 6.8), enzyme extract, (5%) pyrogallol, H₂O₂ (0.5%) and distilled water. Absorbance was measured upto 5 min at 420 nm. One unit of POD activity is defined as μmol of purpurogallin formed μg^{-1} protein min^{-1} .

3.8.3 Catalase (CAT)

Catalase (CAT) activity was calculated according to the method of Egashira et al. (1989) by measuring the level of oxygen release from the dissociation of hydrogen peroxide in darkness. 50 mM H₂O₂ in phosphate buffer (50 mM, pH 7.0) were directly added to 1 ml of culture in the reaction vessel fitted with oxygen analyzer (HI 9147) was kept in dark and used to calculate released oxygen due to enzymatic dissociation of hydrogen peroxide for 5 min. One unit of catalase is defined as the amount of enzyme required to produce one nmol O₂ min^{-1} .

3.8.4 Glutathione-S-transferase (GST) activity

The procedure of Habig et al. (1974) was used to measure GST activity. Known amount of cyanobacterial culture was centrifuged and pellet was resuspended in phosphate buffer (3ml, 100mM, pH 6.25). After sonication for 10 min, culture was centrifuged (20,000g, 20min) and

supernatant was used as enzyme extract. Reaction mixture consisted of enzyme extract (0.2 ml), CDNB (0.75mM, 1 ml), Glutathione (1 ml) and phosphate buffer (0.8 ml). The absorbance was recorded at 340 nm due to conjugate formation between CDNB and glutathione. The value of extinction coefficient $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate enzyme activity. One unit of enzyme activity is defined as nmol of CDNB conjugates formed mg^{-1} protein min^{-1} .

3.9 Non enzymatic antioxidants

3.9.1 Glutathione

Glutathione content (GSH) was determined by using the method of Ellman's (1959). Known volume of cyanobacterial culture was centrifuged, washed with distilled water and sonicated in phosphate buffer (0.2M, pH 8.0) for 10 min. The content was again centrifuged and supernatant was used for estimation of glutathione. The reaction mixture consisted of phosphate buffer, 5–5-dithiobis-2- nitro benzoic acid and enzyme extract. The absorbance was measured at 412 nm using glutathione as standard. Glutathione was calculated in terms of $\mu\text{g } \mu\text{g}^{-1}$ protein.

3.9.2 Ascorbate

Ascorbate was estimated by method of Chinnoy's et al. (1976). Culture was centrifuged and cell pellet was resuspended in metaphosphoric buffer (20 μmol , pH5.4) and sonicated and again centrifuged. To known amount of supernatant, 2, 6 dichloroindophenol dye was added and intensity of developed pink colour was measured at 530 nm by using L-ascorbic acid as standard. The ascorbic acid content was expressed in $\mu\text{g } \text{mg}^{-1}$ protein.

3.10 Stress indicators

3.10.1 Superoxide radicals (SOR)

Superoxide radicals (SOR) were measured according to the method of Elstner and Heupel (1976). Cyanobacterial culture was centrifuged and sonicated in potassium phosphate buffer (65mM, pH 7.8). The supernatant obtained after centrifugation was used as cell extract. To this cell extract, hydroxylamine hydrochloride (10mM) and phosphate buffer were added and incubated for 20 min at 25°C. Naphthylethylene diamine dihydrochloride (7mM) and sulfanilamide (17mM) were added to the above reaction cocktail and centrifuged. The

absorbance of aqueous layer was measured at 530 nm using NaNO_2 as standard and SOR was expressed in $\mu\text{mol } \mu\text{g}^{-1}$ protein.

3.10.2 Hydrogen peroxide (H_2O_2)

H_2O_2 content was estimated according to protocol of Velikova (2000). Culture was homogenized in trichloro acetic acid (0.1%) and centrifuged. The reaction mixture contained potassium phosphate buffer (10mM, pH 7.0), cell extract, and KI (1M) solution. Absorbance of reaction mixture was taken at 390 nm using hydrogen peroxide as standard. H_2O_2 content was expressed in $\mu\text{mol mg}^{-1}$ protein.

3.10.3 Lipid peroxidation

Malondialdehyde content was determined by the method of Heath and Packer (1968), which is based on the formation of 2-thiobarbituric–malondialdehyde (MDA-TBA) adduct. Known volume of culture was centrifuged and resuspended in TCA (5%) and homogenized. The homogenate was sonicated and centrifuged. To known amount of extract, TBA (0.5%) was added and heated at 90°C for 30 min. After cooling, content was centrifuged and absorbance was taken at 532 and 600 nm. The value of nonspecific absorption (600 nm) was subtracted from the values recorded at 532 nm. The concentration of malondialdehyde was calculated by using extinction coefficient $155\text{mM}^{-1}\text{cm}^{-1}$ and expressed as $\mu\text{mol mg}^{-1}$ protein.

3.11 Nitrogen source uptake and nitrogen assimilation enzymes

The rate of depletion of nitrate, nitrite, and ammonium from the liquid media was used to measure their uptake by cyanobacteria. Since insecticide interfered with ammonium uptake estimation so, cartap pretreated and non-treated cells were used for nitrogen uptake study. 7 day old cultures were sampled, centrifuged and resuspended in BG11 medium supplemented with different concentration of cartap (5, 10, 20 $\mu\text{g ml}^{-1}$) and incubated for 24 h. Untreated and pretreated cells were harvested and resuspended in media containing different nitrogen sources such as ammonium chloride as ammonium (100 μM), sodium nitrite as nitrite (100 μM), potassium nitrate as nitrate (500 μM) separately and incubated under light (44.5 μE). After specific time intervals, the supernatant was collected and estimated for ammonium, nitrate and nitrite amount. Cyanobacterial cells were used for estimation of nitrogen assimilation enzymes activities.

3.11.1 Nitrite estimation

Nitrite content in supernatant was measured by method given by Nicholas and Nason (1957). 1 ml sulphanilamide (1%) was added to 2 ml of supernatant. After vortexing, 1 ml of N-(1-Naphthyl) ethylenediamine hydrochloride solution was added and incubated for 15 min for development of pink coloured product whose intensity was measured at 540 nm spectrophotometrically considering sodium nitrite as standard. The nitrite uptake was expressed as μmol nitrite uptake μg^{-1} protein.

3.11.2 Nitrate estimation

The nitrate was measured by modified method of Robinson et al. (1959) where chloroform was replaced by glacial acetic acid in solution of brucine. 2 ml of supernatant was added to 0.5 ml of brucine (5%) and shaken vigorously. To this mixture, 4 ml of conc. H_2SO_4 was added from burette dropwise at constant speed with constant shaking. The absorbance of coloured cooled mixture was recorded at 410 nm and was expressed as μmol nitrate uptake μg^{-1} protein. Potassium nitrate was used as standard.

3.11.3 Ammonium estimation

Ammonium estimation was done by using protocol of Solorzano (1969). For measuring amount of ammonium, the supernatant was diluted five times (1 ml supernatant + 4 ml water). The reaction cocktail contained supernatant (5 ml), sodium nitroprusside (0.2 ml) and oxidizing solution (1 ml). After incubation for 1h, the absorbance of coloured product (blue) was recorded at 660 nm and was expressed as μmol of ammonium up take μg^{-1} protein. Standard graph was plotted by using ammonium chloride.

3.11.4 Nitrate reductase (NR) and Nitrite reductase (NiR) activities

Whole cell Nitrate and nitrite reductase activities were measured according to Herrero et al. (1981). Known volume of cyanobacterial culture was centrifuged and washed with distilled water before resuspending in bicarbonate buffer (0.1 mM). 20 μl of toluene was added and mixed homogenously. This suspension is further used as enzyme extract. The reaction cocktail consisted of enzyme extract (0.2 ml), bicarbonate buffer (0.5 ml), KNO_3 (0.1 ml, 20 μmol), methyl viologen (0.1 ml, 4 μmol) and sodium dithionate (0.1 ml, 10 μmol). After incubation at 37°C for 30 min, nitrite formed was estimated. Nitrate activity was expressed as nmol nitrite formed μg^{-1} protein min^{-1} . When nitrite reductase activity was assessed, KNO_3

was replaced by NaNO_2 , while whole procedure remained same. The amount of nitrite decrease was estimated and was expressed as $\text{nmol nitrite mg}^{-1} \text{ protein min}^{-1}$.

3.11.5 Glutamine synthetase (GS) activity

The GS activity was measured by the protocol of Shapiro and Stadman (1970). Glutamine synthetase activity was measured using whole cyanobacterial cells. Known volume of suspension was centrifuged, washed with distilled water before suspending in imidazole buffer (50mM, pH7). 20 μl toluene was added to 1 ml of suspension and agitated to get enzyme extract. Reaction cocktail contained imidazole buffer, assay mixture (1 ml) and enzyme extract (0.4 ml). After incubation of 1 h at 37°C , stop mixture (4 ml) was added to it and centrifuged. The absorbance of brown colour was recorded at 540 nm. Standard curve was constructed using L (glutamic acid γ -mono hydroxamate). One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of γ - glutamyl hydroxamate min^{-1} protein.

3.12 Total nitrogen estimation

Total nitrogen estimation was done by method given by Piper, (1960). Known amount of algal sample was digested with concentrated H_2SO_4 in Kjeldahl digestion tube at room temperature for 30 min. To this sodium thiosulphate was added and allowed to stand for another 30 min. After that catalyst mixture (10 g anhydrous sodium sulphate and 1g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was added into the flask and glass beads were added to prevent bumping. Digestion was continued for 1h until the digest get clear. Now tube along with contents was placed for automatic distillation by Kjeldahl unit. To this digest, 150 ml distilled H_2O was added, cooled and again added 40% NaOH along the sides of Kjeldahl flask and was connected to the distillation system. Evolved ammonia was collected in 25 ml boric acid in flask containing mixed indicator (0.5 g of Bromophenol green and 0.1g of methyl red indicator in 100 ml of ethanol). This was followed by titration against 0.01N sulphuric acid. Total nitrogen was calculated by using the following equation:

$$\text{Total N (\%)} = \frac{[(T - B) \times \text{Normality of H}_2\text{SO}_4 \times 1.4 \times 300]}{\text{Weight of sample}}$$

Where T is titer value for sample and B is for blank

3.13 Osmolyte

3.13.1 Proline

Proline content was estimated by the method of Bates et al. (1973). Known amount of cyanobacterial culture was centrifuged, washed with distilled water, resuspended in aqueous sulphosalicylic acid (3%). The contents were sonicated, centrifuged and to supernatant, glacial acetic acid and acidic ninhydrin were added. The mixture was then placed in boiling water bath for 1 h and the reaction was terminated by shifting test tubes to an ice-bath and then toluene was added. After vortexing, absorbance of toluene layer was measured at 520 nm using proline as standard.

3.13.2 Sucrose

Sucrose was measured by anthrone reagent using protocol given by Van Handel (1968). Culture was homogenized in 80% ethanol and placed in boiling water bath to destroy hexoses. After cooling, potassium hydroxide (30%) was added and incubated in boiling water bath. Anthrone reagent was added to tubes after cooling and incubated for 20 min at 38°C. Intensity of coloured product was measured at 620 nm using sucrose as standard.

3.14 Cartap uptake study

Cartap uptake study by cyanobacterial culture was done by growing it in nitrate depleted culture media supplemented with 5-30 $\mu\text{g ml}^{-1}$ of cartap hydrochloride. 7 day old culture (Exponentially growing) culture was inoculated to get initial absorbance 0.5 at 720 nm. After specific time interval, sample was collected and cartap was estimated in both cells and supernatant. Supernatant was used without any treatment but cells were first washed twice with distilled water and sonication was done to disintegrate cells. This mixture is now centrifuged (20,000g) and used for estimation of intracellular cartap by method given below.

3.14.1 Cartap estimation

The method published by Bureau of Indian Standards, New Delhi (1994) in their report of Indian standards cartap hydrochloride technical- specification was used to measure amount of cartap in the sample. 2 ml buffer was added to 2 ml of sample and incubated at 35°C for 20 min. The absorbance of yellow coloured product was recorded at 412 nm. Cartap hydrochloride was used to make standard graph.

3.15 Field emission scanning electron microscopy (FESEM)

The cyanobacterial cells were treated with different concentrations of cartap ranging from 5-20 $\mu\text{g ml}^{-1}$. After the 3rd, 6th and 9th day, the samples were subjected to SEM analysis (Martínez and Martínez, 2018). The sample was first dried and then coated with platinum. Hitachi SU 8000 scanning electron microscope was used for imaging the sample. Small working distance (8.2 mm) and low excitation voltage (5.0 kV) were used for imaging. Under these experimental conditions, it was not necessary to metalize the samples because charging effects were minimal and true sample features were not masked.

3.16 Synthesis of chitosan/tripolyphosphate nanospheres

Chitosan solution (0.01% w/v, pH 6) was prepared by adding chitosan in 0.1% (w/v) acetic acid and kept on overnight shaking until fully dissolved and insecticide cartap was added in it. Sodium tripolyphosphate (TPP) 0.1% (w/v) (pH 4.5 and 8°C) was poured dropwise to the chitosan solution under magnetic stirring at 1000 rpm. The nanospheres started forming spontaneously when TPP was added to the chitosan cartap mixture as it acts as crosslinker and initiates the process of ionic gelation. The mixture was agitated overnight. The final concentration of cartap hydrochloride was 1 mg ml^{-1} in the formulation. The blank chitosan tripolyphosphate nanospheres (CS-TPP nps) were prepared similarly without adding insecticide (Gan and Wang, 2007; Gan et al., 2005; Qi et al., 2004; Prabakaran and Mano, 2005). Chitosan nanospheres were collected by centrifugation at 8000 rpm for 15 min.

3.17 Chitosan/sodium alginate nanospheres

10 ml solution of sodium alginate (0.6 mg ml^{-1}) was prepared and insecticide cartap hydrochloride (0.5 mg ml^{-1}) was mixed with it. 2 ml solution of calcium chloride (0.67 mg ml^{-1}) was added drop wise to the above solution with help of syringe (slowly). Resulting calcium alginate pre gel maintained under proper stirring for 1h. 2 ml solution of chitosan (0.3 mg ml^{-1} , pH 6) prepared in 1% (w/v) acetic acid was poured dropwise to the pre gel under magnetic stirring at 1000 rpm and the mixture was kept under stirring overnight to enable the formation of nanospheres. The blank chitosan alginate nanospheres (CS-ALG nps) were prepared similarly without adding insecticide (De and Robinson, 2003). Chitosan nanospheres were collected by centrifugation at 8000 rpm for 15 min.

3.18 Physicochemical characterization

Cartap chitosan tripolyphosphate nanospheres (C-CS-TPP nps) were characterized by dynamic light scattering (DLS) ZS90 instrument. The phenomena of dynamic light scattering (DLS) were utilized to determine the average size of the nanospheres and their polydispersity index. All the DLS analyses were made in triplicate. The polydispersity and zeta potential of the nanospheres were determined by using a Zetasizer (Malvern Instruments).

The stability of the suspensions, with and without the insecticide, was evaluated by measuring the average particle diameter, polydispersity, and zeta potential during a period of 30 days, with the samples stored in amber flasks at ambient temperature (25°C).

3.18.1 Fourier transforms infrared (FTIR)

The structural features of nanospheres were studied by FTIR spectra of chitosan, cartap, blank CS-TPP and C-CS-TPP nanospheres. KBr disc method was used to record the spectra in spectral region between 4000 and 400 cm^{-1} by using Perkin Elmer - Spectrum 400 FT-IR/FIR Spectrometer having resolution of 1 cm^{-1} .

3.18.2 Field emission Scanning Electron Microscopy

Field emission scanning electron microscope (FESEM) was employed to study the morphology of synthesized chitosan nanospheres. Platinum coated powder sample was used to examine the surface topography by a Hitachi SU 8010 scanning electron microscope operated at an applied voltage of 5 KV.

3.18.3 X-ray Diffraction

X-ray diffractometer ((XPERT-PRO, Analytical) was used to study the diffraction patterns of cartap, chitosan, CS-TPP and C-CS-TPP nanospheres. The samples were irradiated with monochromatized Cu K α radiation (1.542 Å) and analyzed between 5 and 70- (2θ) at voltage (45 kV) and current (40 mA).

3.19 Encapsulation Efficiency

Encapsulated insecticide amount was calculated by centrifugating the insecticide loaded nanospheres at 20,000 g (20 min). The supernatant was used to determine the amount of free insecticide spectrophotometrically by using calibration curve of free insecticide. The encapsulation efficiency of nanospheres were calculated as follows:

$$\text{Encapsulation efficiency (\%)} = \left[\frac{\text{Total amount of insecticide} - \text{Free insecticide}}{\text{Total amount of insecticide}} \right] * 100$$

3.20 Release kinetics assay

The *in vitro* release of cartap from nanospheres was done by using two compartment system which was maintained under continuous agitation (Grillo et al., 2010). 1 ml sample was added in dialysis membrane (donor) and placed in acceptor compartment. Dialysis membrane acts as separator to separate donor compartment from the acceptor compartment. Only free insecticide was allowed to pass from membrane and insecticide nanospheres were retained. Samples were taken at different time intervals from the acceptor compartment for spectrophotometric analysis. All the measurements were performed in triplicate. The mechanisms of release of the insecticide from nanospheres were evaluated using the zero order, first order, Higuchi, Korsmeyer-Peppas and Hixson mathematical models (Higuchi, 1961; Korsmeyer et al., 1983; Costa and Lobo, 2001).

3.21 Statistical analysis

All the values presented are the mean of three independent experiments $\pm$ standard error (SE). Statistical analysis of data was done by applying two-way analysis of variance test using Graph Pad Prism 8.0 version 8.3.0 followed by Tukey's honest significance difference test.

Eco-toxicological effects of pesticides on cyanobacteria are of great concern. Effect of cartap was studied on stress response and physiological parameters of *Anabaena variabilis* ARM 441. Chitosan tripolyphosphate and chitosan alginate nanospheres loaded with cartap hydrochloride were fabricated for its controlled delivery.

4.1 Effect of cartap hydrochloride on growth and physiological parameters of heterocystous cyanobacteria

4.1.1 Growth of *Anabaena variabilis* ARM 441 in the presence of cartap

The influence of cartap on *Anabaena variabilis* growth (dry biomass) and heterocyst frequency was investigated since these are significant growth indicators that reflect the condition of cyanobacteria growth and development.

Growth of *Anabaena variabilis* at graded concentrations (0, 5, 10, 20 $\mu\text{g ml}^{-1}$) of cartap is depicted in Fig. 4.1. Growth increased from 0.101 on zero day to 0.656 mg ml^{-1} on day 21 in absence of cartap. However, concentration-dependent decrease in growth was observed in presence of cartap. The cyanobacterium survived up to 30 $\mu\text{g ml}^{-1}$ cartap and did not grow above 40 $\mu\text{g ml}^{-1}$ cartap. At 40 $\mu\text{g ml}^{-1}$ cartap, pigments were released in the medium due to cell lysis resulting in a colorless culture. The growth curve indicated that the organism exhibited lag phase up to 6 days followed by gradual increase and reached to a steady state on the 16th day in control, whereas in pesticide treated samples the growth was maximum on the 18th day. The growth declined up to 30.93 in 5 $\mu\text{g ml}^{-1}$, 57.31 in 10 $\mu\text{g ml}^{-1}$ and 96.99% in 20 $\mu\text{g ml}^{-1}$ of cartap treated samples compared to the control on day 21 of insecticide treatment.

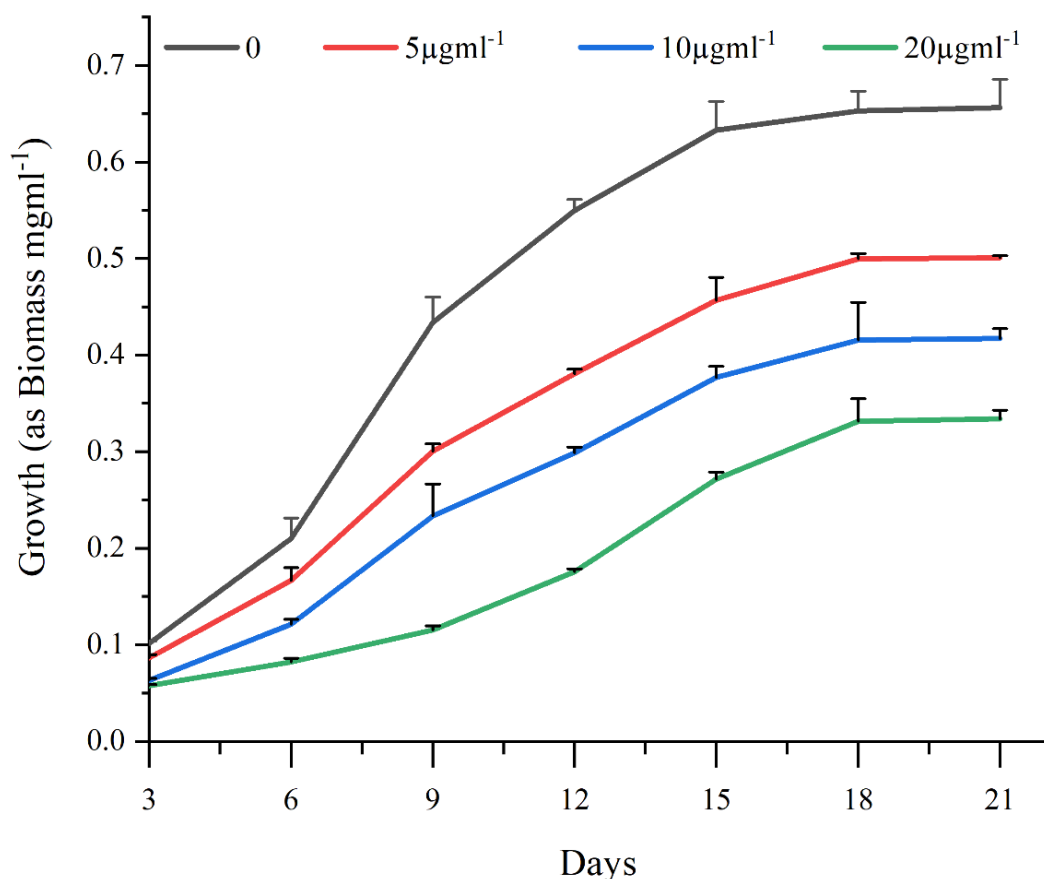


Fig. 4.1: Effect of different concentration of cartap on growth (dry biomass) of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE.

Cartap effect on specific growth rate and generation time was also studied (Fig. 4.2). In control cultures cyanobacterium showed specific growth rate constant 0.243 d^{-1} and generation time of 2.85 h. In 5, 10 and $20 \mu\text{g ml}^{-1}$ the specific growth rate constant was 0.219, 0.217, 0.117 d^{-1} while showing generation time of 3.16, 3.19 and 5.92 h. With increase in insecticide dose, specific growth rate decreased, while increasing generation time of cyanobacterium.

The heterocyst frequency in control culture was 14.83 on day 7, which was increased to 15.8 %. In case of cartap treated cultures, due to cartap stress there was decrease in heterocyst frequency in a dose dependent manner. In 5, 10, $20 \mu\text{g ml}^{-1}$ cartap treated cultures, the heterocyst frequency was 12.8, 11.9 and 10.06 after 7 days of incubation (Fig.4.3). Absorption spectra of *Anabaena variabilis* was depicted in Fig.4.4 on the day 21. Cartap treated cyanobacterial cultures showed significant reduction in the peak height of

photosynthetic pigments as compared to the control. The peak height of spectra decreased in dose dependent manner.

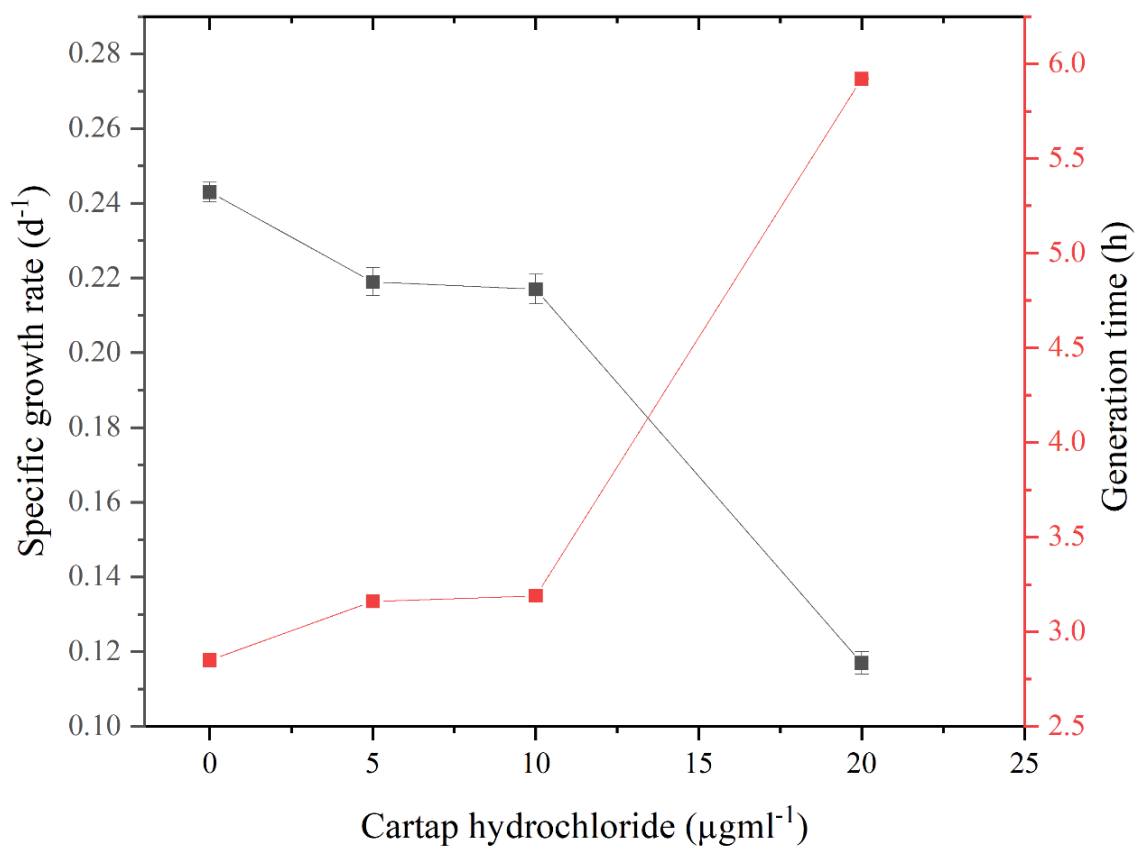


Fig. 4.2: Effect of cartap on the specific growth rate and generation time of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE.

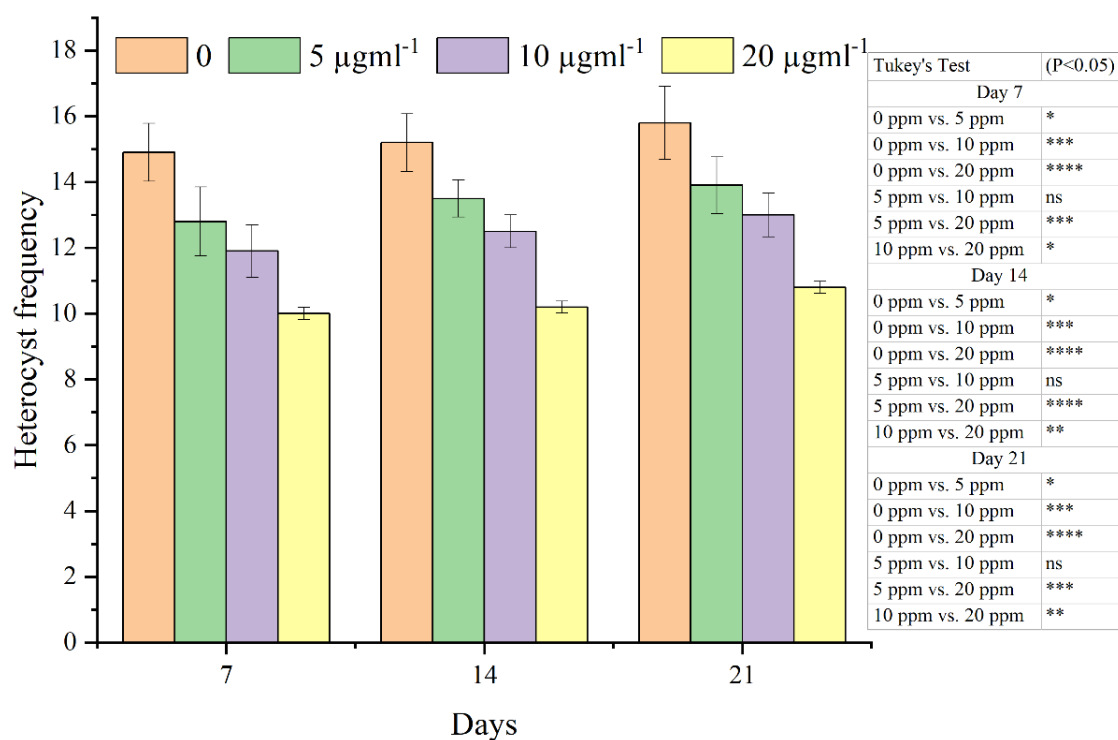


Fig. 4.3: Effect of cartap on heterocyst frequency of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE.

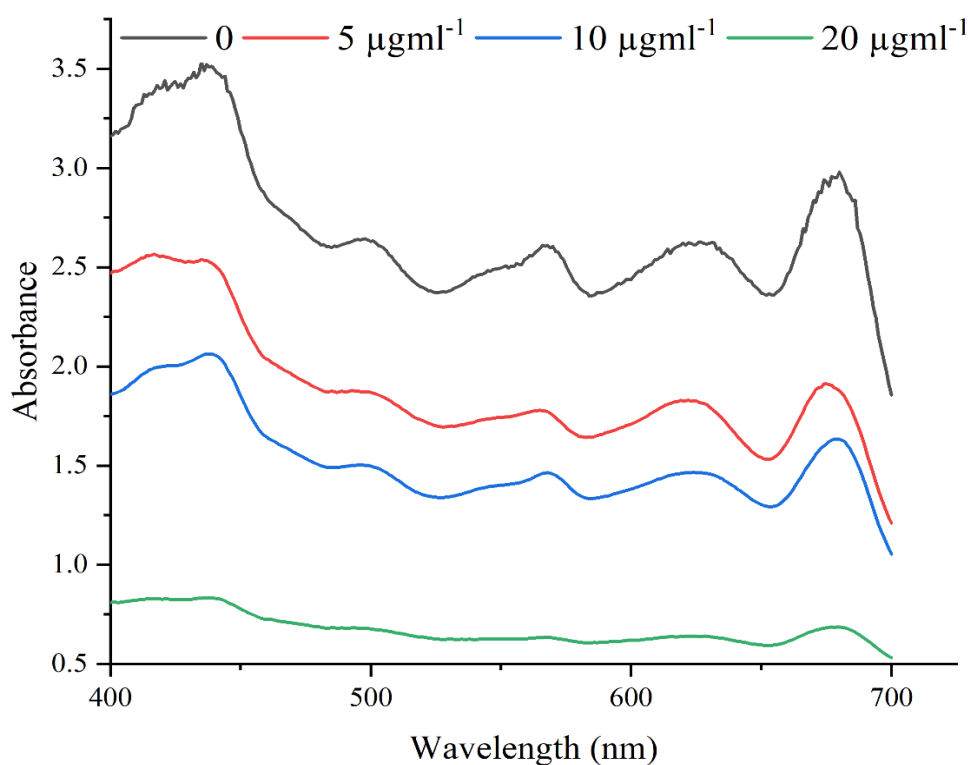


Fig. 4.4: Effect of cartap on the absorbance spectra of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE.

Decrease in growth rate and generation time suggested that cartap had an effect on the microorganism's cell division as well. *Nostoc muscorum*, *Arthrospira fusiformis*, *Microcystis aeruginosa*, *Spirulina platensis*, *Leptolyngbya boryanum* and *Anabaena sp.* on exposure to increasing concentration of glyphosate (0.01-100mM), there was decrease in specific growth rate constant (Forlani et al., 2008). Chloripyrifos treated *Synechocystis sp.* PUPCC 64 showed concentration-dependent increase in generation time from 38 h at 2.5 $\mu\text{g ml}^{-1}$ to 100 h at 12.5 $\mu\text{g ml}^{-1}$ (Singh et al., 2011).

Similar observations, were reported when low dose of glyphosate (0.2 mM) did not affect the biomass production in *Spirulina sp.* as compared to higher dose (20mM) (Lipok et al., 2007). Effect of three different enantiomers of diclofop methyl (DM) (DM, R-DA and S-DA) was studied on *Microcystis aeruginosa* which resulted in growth inhibited in dose dependent manner by all the three forms (Ye et al., 2014). *Phormidium foveolarum* reported 6% and 15% decline whereas *Nostoc muscorum* showed 41% and 50% inhibition when grown in 10 and 20 $\mu\text{g ml}^{-1}$ of oxyfluorfen (Sheeba et al., 2011). Low concentration of malathion varying between 0.02-20 $\mu\text{g ml}^{-1}$ increased the biomass in *Spirulina platensis*, *Anabaena oryzae* and *Nostoc muscorum*, while high concentration of 50 and 100 $\mu\text{g ml}^{-1}$ inhibited the growth (Ibrahim et al., 2014).

Response of cyanobacteria to different pesticides has been shown to be stimulatory on growth at low doses and inhibitory at higher concentrations. Inhibitory effect of different insecticides was reported on *Synechocystis sp.* (Singh et al., 2011), *Anabaena sp.* (Shen et al., 2009), *Leptolyngbya foveolarum* (Singh et al., 2014), *Tolypothrix scytonemoides* (Rajendran et al., 2007), *Nostoc* (Chen et al., 2007), *Phormidium valderianum* (Palanisami et al., 2009), *Anabaena sphaerica* (Chakraborty et al., 2017), *Fischerella sp.* (Tiwari et al., 2018) and *Anabaena* PCC 7120 (Tiwari et al., 2016).

There was significant reduction in growth of *Nostoc humifusum*, when treated with increasing concentrations of malathion (Poliyedath and Shamina, 2019). Growth was retarded in *Microcystis sp.* and *Chroococcidiopsis sp.*, at 5-500 $\mu\text{g ml}^{-1}$ of deltamethrin and cypermethrin (Bano et al., 2021). Reduced growth was observed with increase in dose of methyl parathion in *Fischerella sp.* (Tiwari et al., 2018). The growth of *Anabaena sphaerica* was reduced by 3.13, 9.4, 26.83, 66.4% at 5, 10, 20, 30 $\mu\text{g ml}^{-1}$ of malathion after 12 days of incubation (Chakraborty et al., 2017). The inhibition in growth of the alga might have been due to the

inhibition in photosynthetic and other physiological activities (Manikar et al., 2013; Bhattacharya et al., 2011).

Different species of cyanobacteria exhibits variable response to insecticides mainly due to their inherent capacity to detoxify them or the habitat from which they are isolated (Singh et al., 2014; Singh et al., 2013; Tiwari et al., 2016). In present study, dose dependent decrease in heterocyst frequency was observed after 21 days of incubation. Heterocyst frequency was inhibited in concentration dependent manner in *Anabaena sphaerica* upon malathion exposure (Chakraborty et al., 2017). *Plectonema boryanum*, *Aulosira fertilissima*, *Anabaena variabilis* and *Nostoc muscorum* growth was inhibited due to endosulfan treatment. This decrease in growth was due to chlorophyll synthesis and photosynthesis inhibition (Kumar et al., 2008). Toxicity of malathion at higher concentration in *A. variabilis* was found to be due to the presence of thin and diffluent mucilage and increased permeability of pesticide across the cell membrane (Manikar et al., 2013).

On the basis of above-mentioned results, three concentrations of cartap 5 $\mu\text{g ml}^{-1}$ (half of recommended dose), 10 $\mu\text{g ml}^{-1}$ (recommended dose) and 20 $\mu\text{g ml}^{-1}$ (double of recommended dose) were selected for further experimentation.

4.1.2 Protein and carbohydrate content

Cyanobacteria responded to stress induced by cartap by inhibiting protein synthesis in a dose dependent manner. In control culture, the amount of protein was 69.09 $\mu\text{g protein ml}^{-1}$ whereas in treated culture it was decreased to 70.38, 65.45, 55.14 $\mu\text{g protein ml}^{-1}$ at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap after 7 days of incubation. At the end of 21 days, the cyanobacterium exhibited 64.56, 61.63 and 50.69 $\mu\text{g protein ml}^{-1}$ indicating 6.5, 10.79, 26.63% inhibition at 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively (Fig. 4.5).

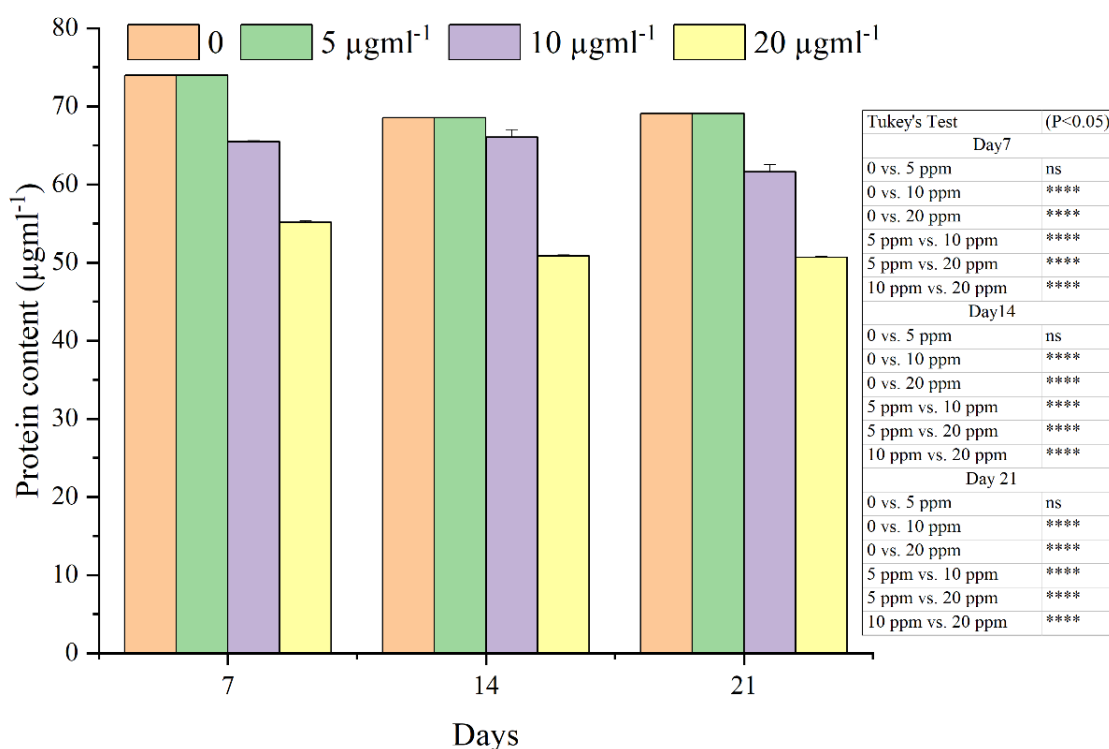


Fig. 4.5: Effect of cartap on the protein content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Cypermethrin ($0.005 \mu\text{g ml}^{-1}$) caused significant augmentation of protein content (35-47%) in *Chattonella marina*, *Skeletonema costatum* and *Scrippsiella trochoidea* within 12 h of exposure while higher concentration (0.05 and $0.1 \mu\text{g ml}^{-1}$) decreased the protein synthesis in *Chattonella marina* and *Scrippsiella trochoidea* without affecting protein synthesis in *Skeletonema costatum* (Wang et al., 2011). *Oscillatoria tenue* and *Anabaena cylindrica* when treated with diazinon for 24 days resulted in inhibited protein synthesis at higher dose of 5 - $10 \mu\text{g ml}^{-1}$ while enhanced protein synthesis was observed in 1 - $3 \mu\text{g ml}^{-1}$ (Ghadai et al., 2010a). Endosulfan inhibited the protein content of *Nostoc muscorum*, *Anabaena fertilissima*, *Nostoc calcicola* and *Anabaena variabilis* (Shehata et al., 2001; Kumar et al., 2008a; Prasad et al., 2011). Protein content of *Chroococcidiopsis* sp. and *Microcystis* sp. was increased by 0.8 and 0.5% respectively, when treated with cypermethrin however, treatment with deltamethrin resulted in decreased protein amount by 4.4 and 1.8% respectively (Bano et al., 2021). Dose dependent reduction was seen in protein content of *Anabaena sphaerica* culture when treated with malathion dose ranging between 5 - $30 \mu\text{g ml}^{-1}$ (Chakraborty et al., 2017).

However, in contrast to protein, carbohydrate content of cartap treated cultures were increased in dose dependent manner. In untreated culture, the carbohydrate content increased from 2.0 to 13.86 $\mu\text{g ml}^{-1}$ from day 7 to day 21 (Fig. 4.6). In 5, 10 and 20 $\mu\text{g ml}^{-1}$ cartap treated culture,

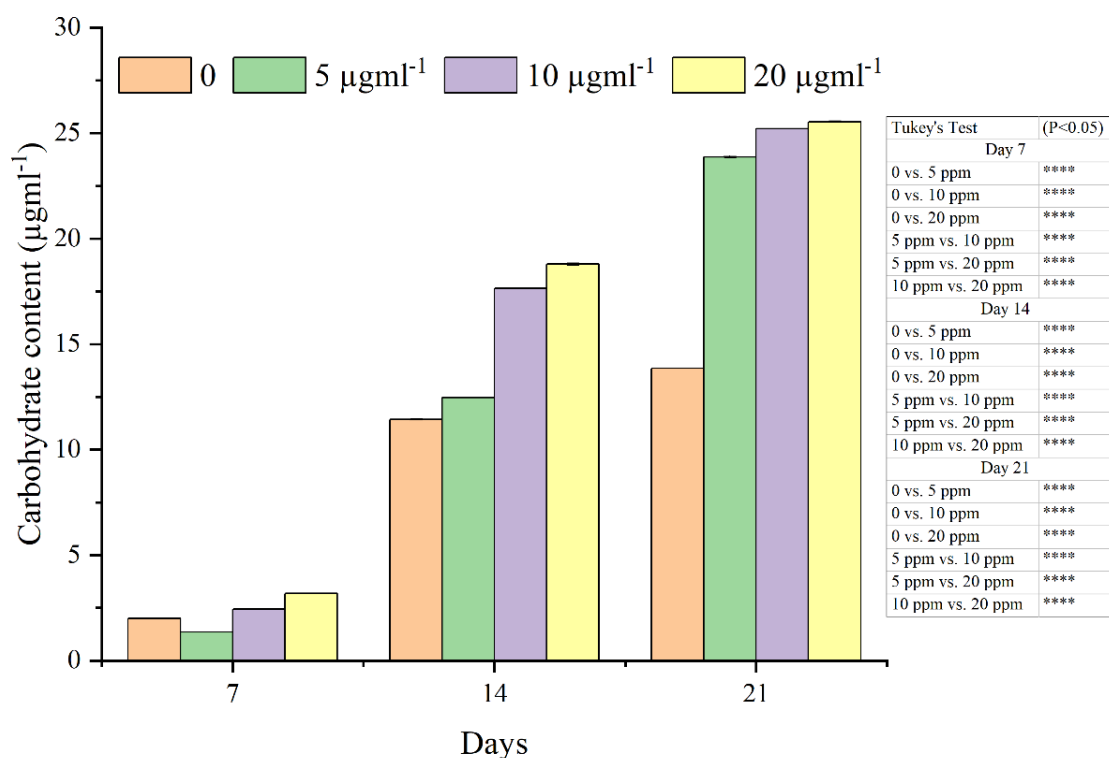


Fig. 4.6: Effect of cartap on the carbohydrate content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

amount of carbohydrate accumulation was 1.34, 2.44, 3.19 $\mu\text{g ml}^{-1}$ at the end of 7 days, which was increased to 23.87, 25.02 and 25.53 $\mu\text{g ml}^{-1}$ after 21 days indicating 72.27, 81.87, 84.25% increase in carbohydrate synthesis. The increased sugar content could be an adaptation approach to help the organism survive in a cartap stressed environment.

The total carbohydrate content of *Anabaena variabilis* was also significantly increased by 40, 61, 68 and 84% in 25, 50, 75 and 100 $\mu\text{g ml}^{-1}$ due to malathion stress (Manikar et al., 2013). Due to stress when protein synthesis was suppressed then algal cells depending on their genotype either synthesize carbohydrate or lipid to cope up with stress. Disturbances in nitrogen metabolism and photosynthetic activity could lead to imbalanced cell composition.

(Manikar et al., 2013). With trichlorofon and thiobencarb, a similar trend was observed in *Anabaena* PCC 7119 and *Anabaena variabilis* (De et al., 2014; Qian et al., 2009). Carbohydrate content of *Fisherella* sp. was reduced by 59.6% after 8 days of treatment with methyl parathion (Tiwari et al., 2018). Concentration dependent inhibition was seen in carbohydrate content of *Anabaena sphaerica* culture when treated with malathion dose ranging between 5-30 $\mu\text{g ml}^{-1}$ (Chakraborty et al., 2017).

4.1.3 Photosynthetic Pigment

The production of photosynthetic pigments was inhibited in the same way as growth of *Anabaena variabilis* ARM 441 upon cartap treatment. The synthesis of photosynthetic pigments was checked for 21 days at an interval of 7 days. Chlorophyll *a* at the end of day 7 showed decreasing trend from 37.22 to 98.73% and was 5.043, 4.18, 3.39 and 2.54 $\mu\text{g ml}^{-1}$ at 0, 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap (Fig. 4.7), whereas carotenoids decreased by 30.55, 47.22 and 72.22%, respectively (Fig. 4.8). Similarly, phycocyanin (PC) was decreased by 29.41, 31.37 and 70.58% (Fig.4.9b), allophycocyanin (APC) by 28.33, 40.26 and 49.35% (Fig. 4.9a), and phycoerythrin (PE) by 63.47, 72.17 and 86.95% (Fig. 4.9c) at 5, 10 and 20 $\mu\text{g ml}^{-1}$ cartap, respectively. At recommended dose of cartap (10 $\mu\text{g ml}^{-1}$), photosynthetic pigments showed decline in the order of Chl *a* >APC>PC>Car>PE on day 7. At 20 $\mu\text{g ml}^{-1}$ cartap, which is two times of field dose, levels of all the photosynthetic pigments were decreased from 28-98%. This suggests that insecticide showed inhibitory effect on the synthesis of photosynthetic pigments in a dose dependent manner.

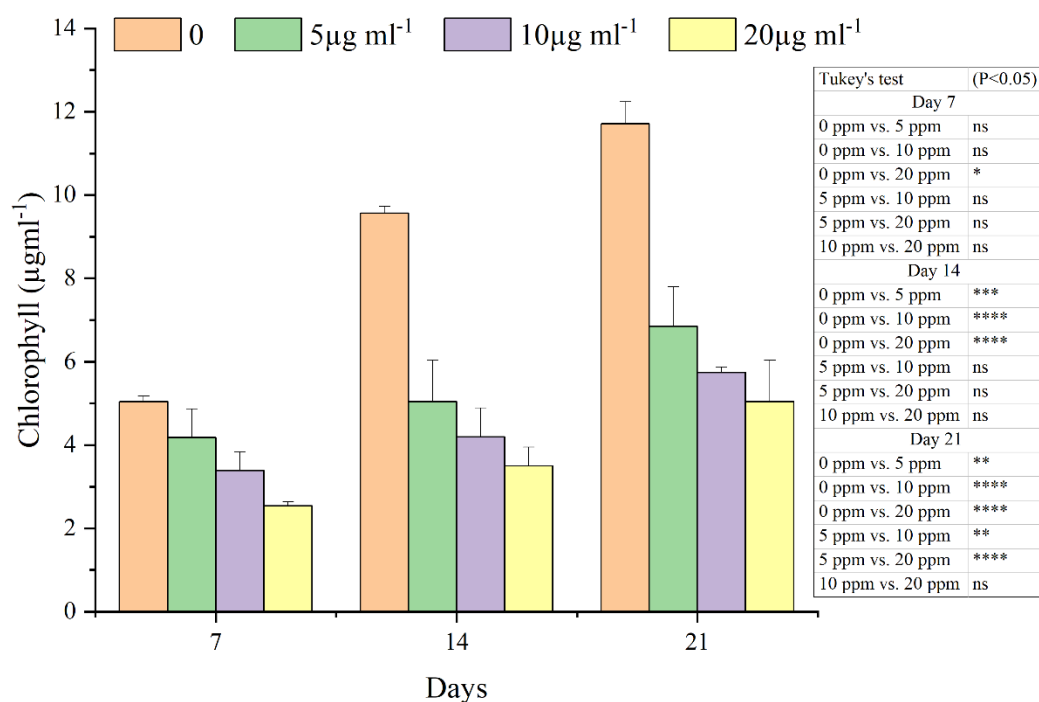


Fig. 4.7: Effect of cartap on chlorophyll content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

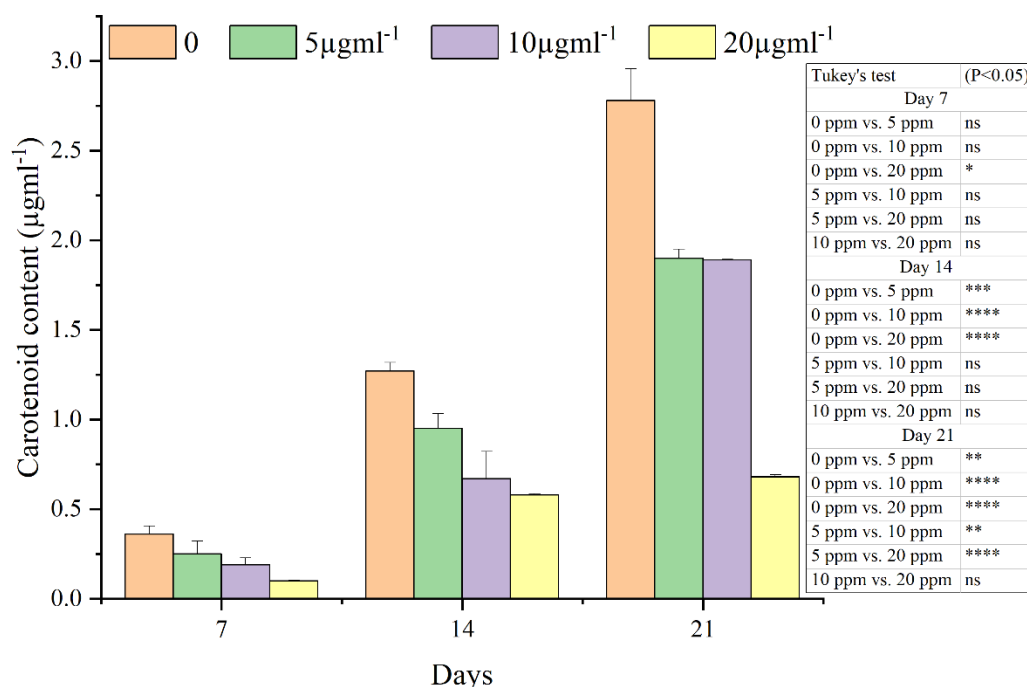
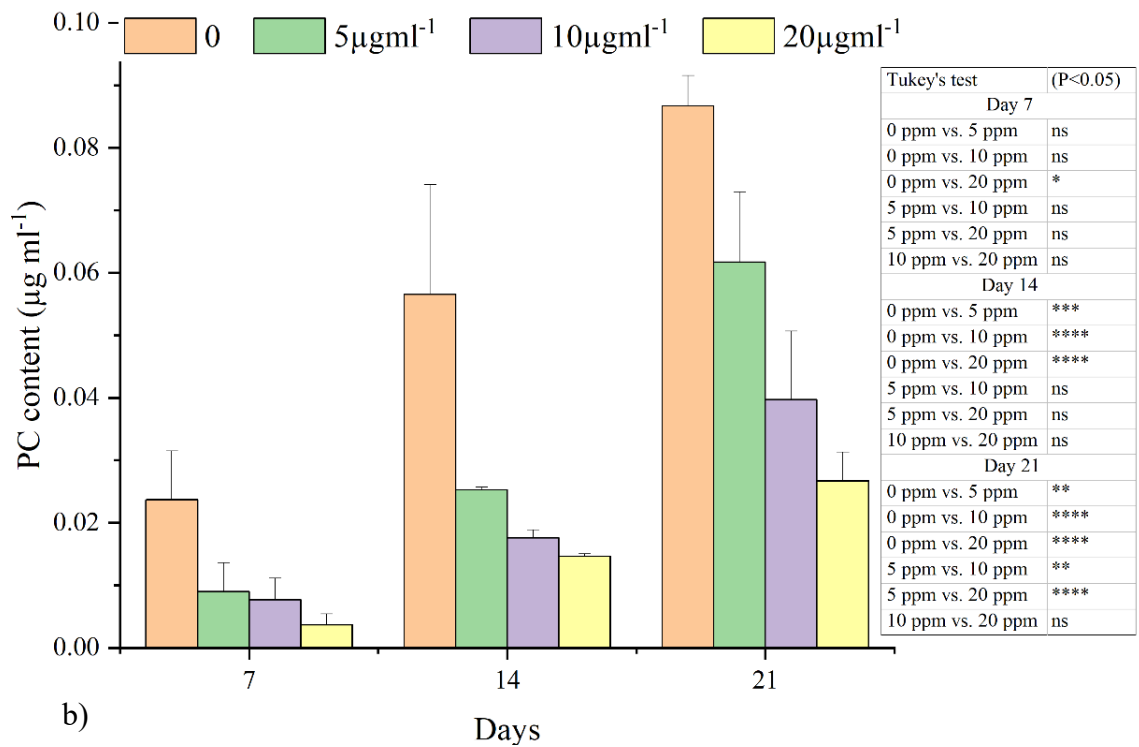
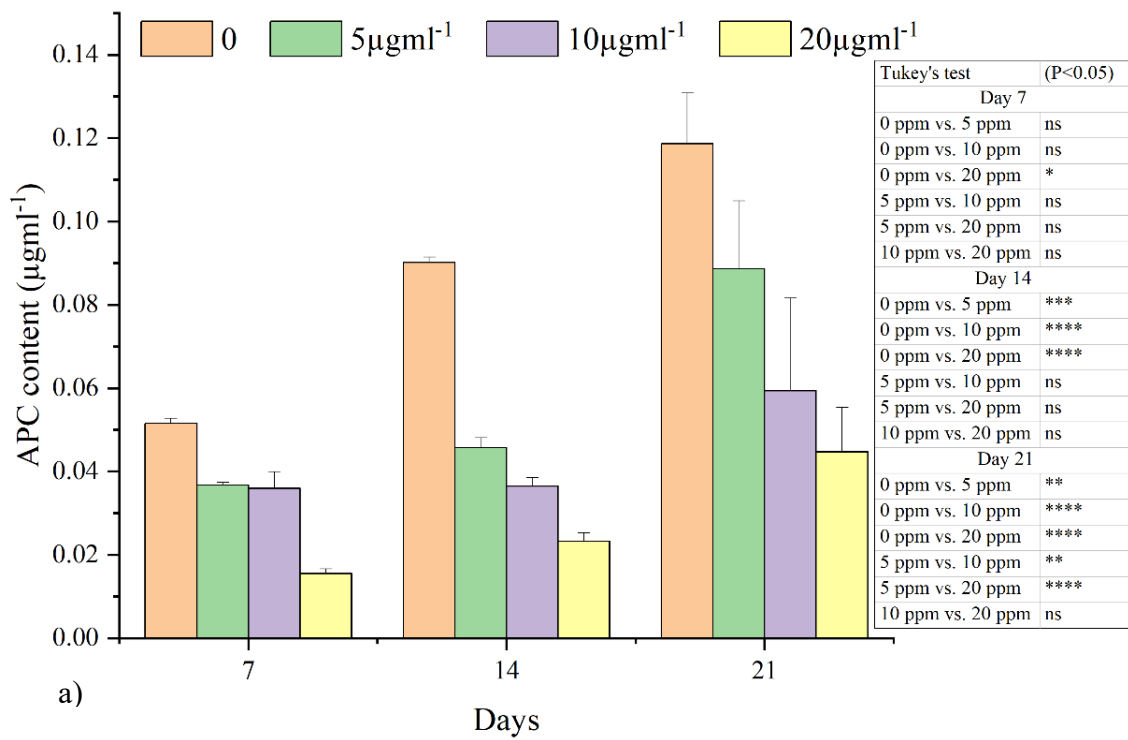


Fig. 4.8: Effect of cartap on carotenoids content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).



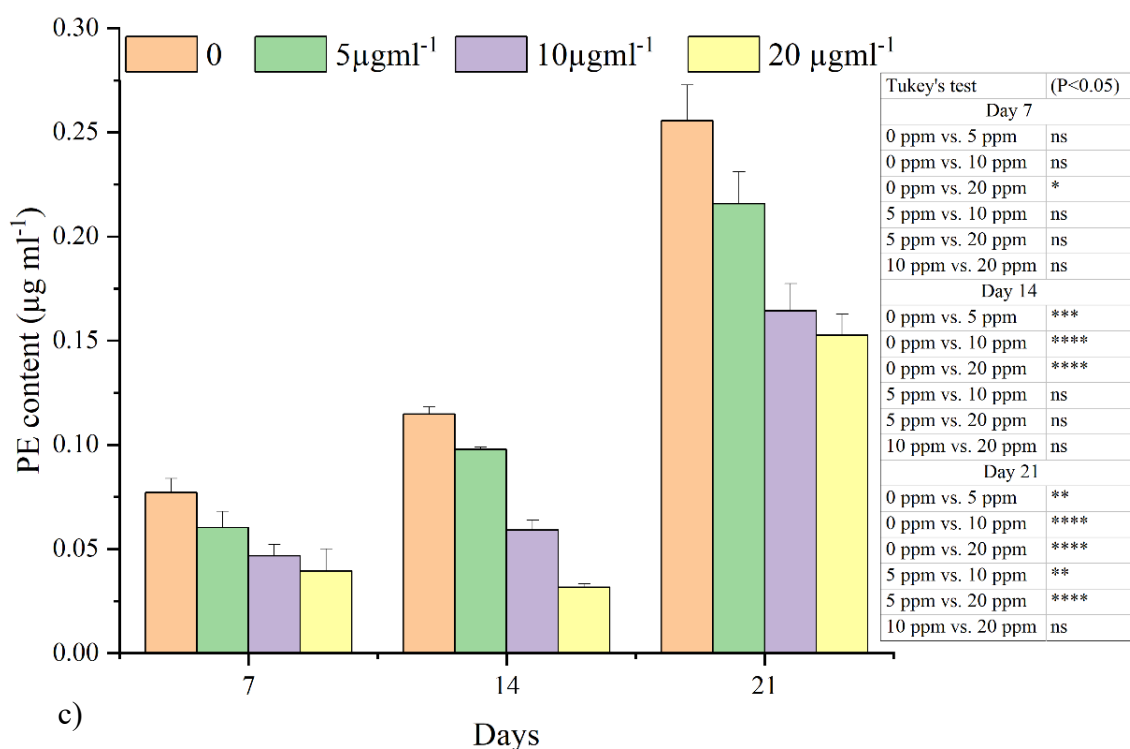


Fig. 4.9: Effect of cartap on photosynthetic pigments of *Anabaena variabilis* ARM 441. a) Allophycocyanin (APC); b) phycocyanin (PC); c) phycoerythrin (PE). Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Growth inhibition of *Anabaena variabilis* ARM 441 with increase of cartap concentration can be explained on the basis of damaging effects of insecticide on photosynthetic pigments (Bhattacharyya et al., 2011). Our results showed that cartap inhibited the production of all photosynthetic pigments in a dose dependent manner (Fig. 4.7, 4.8, 4.9). The deleterious effect was more pronounced on chlorophyll compared to carotenoids. Such decrease in chlorophyll and carotenoid contents could be attributable to the pigment synthesis inhibition directly by pesticide or increased pigment degradation due to accelerated ROS production at various sites of the photosynthetic electron transport chain during stress (Neha, 2014).

As most of the carotenoids synthesizing enzymes are membrane-bound, reduction of carotenoid biosynthesis might be due to the interaction of the insecticide with these enzymes (Neha, 2014). Decrease in the growth rate of cyanobacteria in presence of insecticide may be due to a decrease in photosynthesis especially chlorophyll-a. Similarly, all photosynthetic pigments of *Nostoc* were inhibited when treated with dimethoate, butachlor and bensulfuron-methyl (Rajendran et al., 2007).

Methyl parathion inhibited chlorophyll and carotenoid content of *Nostoc punctiforme* in concentration and time dependent manner (Bhattacharyya et al., 2014). Chlorophyll *a* and carotenoid content in 20 $\mu\text{g ml}^{-1}$ of malathion treated culture of *Nostoc humifusum* was significantly reduced with respect to control cultures (Poliyedath and Shamina, 2019).

Microcystis sp. and *Chroococcidiopsis* sp. when treated with 5-500 $\mu\text{g ml}^{-1}$ of deltamethrin and cypermethrin inhibition of chlorophyll *a* and carotenoid content in dose and time dependent manner was observed (Bano et al., 2021). Sharp increase was seen in carotenoid content in *Anabaena sphaerica* culture when treated with malathion upto 20 $\mu\text{g ml}^{-1}$ afterwards it was inhibited (Chakraborty et al., 2017).

Inhibition of synthesis of photosynthetic pigments was also reported in *Oscillatoria simplicissima* (Singh et al., 2010) and *Anabaena torulosa* (Singh et al., 2012a), when treated with anilofos. Cartap treatment to *Leptolyngbya foveolarum* stimulated the synthesis of photosynthetic pigments at lower concentration (20 $\mu\text{g ml}^{-1}$) but inhibited the production of chlorophyll *a* and carotenoids at higher concentration (40 and 60 $\mu\text{g ml}^{-1}$) of insecticide (Singh et al., 2014). The photosynthetic pigments of *Anabaena* sp. and *Scenedesmus* sp. also decreased significantly with respect to increasing profenofos concentration as well as duration of exposure (Neha, 2014).

4.1.4 Effect of cartap on photosynthesis and respiration

Impact of cartap toxicity was assessed in terms of oxygen consumption (respiration) and evolution (photosynthesis) in *Anabaena variabilis* ARM 441 (Fig. 4.10 and 4.11). The results revealed that insecticide acted upon microorganism in dose dependent manner. After 1 day of incubation, control culture produced 245.31 nmol of oxygen μg^{-1} Chl *a* min^{-1} and 195.31, 176.56 and 143.75 nmol of oxygen μg^{-1} Chl *a* min^{-1} was produced at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively (Fig. 4.10).

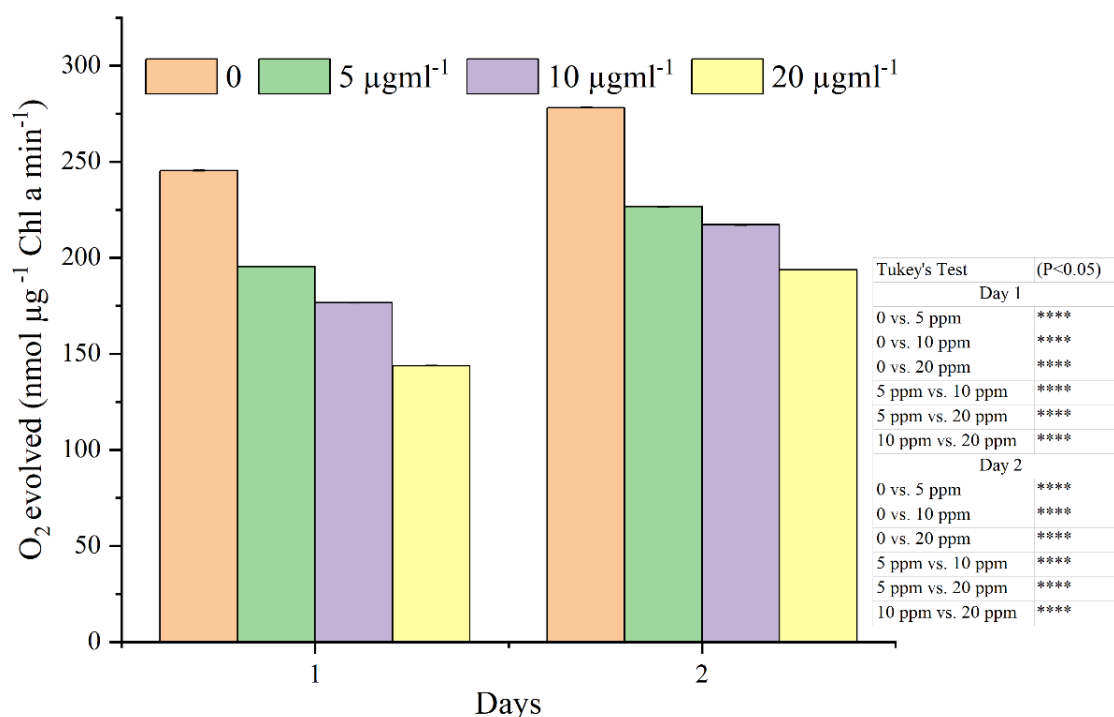


Fig. 4.10: Effect of cartap on photosynthesis of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Rate of evolution of oxygen was decreased as the concentration of cartap increased, producing 226.56, 217.18 and 193.75 nmol of oxygen μg^{-1} Chl *a* min^{-1} at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap after 2 days of incubation corresponding to 18.54, 21.9 and 30.34% decrease respectively.

Photosynthesis plays a key role in the growth and development of photoautotrophs (Prasad et al., 2011). The growth rate of *Anabaena variabilis* was decreased in presence of cartap may be due to low photosynthetic rates. To ascertain correlation between growth rate and photosynthetic rates, photosynthesis and respiration were studied in presence and absence of cartap. In *Anabaena*, effect of cartap on photosynthesis was more pronounced in 20 $\mu\text{g ml}^{-1}$ than 5 and 10 $\mu\text{g ml}^{-1}$. Similar observation was reported by Singh et al. (2014), when *Leptolyngbya foveolarum* was treated with cartap resulted in increased photosynthesis at low concentration (20 $\mu\text{g ml}^{-1}$) but at higher concentration of 60 and 80 $\mu\text{g ml}^{-1}$, photosynthesis was inhibited. 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2, 4 -D also inhibited photosynthesis in *Nodularia*, *Anabaena* and *Nostoc* sp. (Leganes and Fernandez-Valiente (1992). Bensulfuron-methyl (8-10 ml l^{-1}) inhibited photosynthesis by 50% in *Nostoc*

commune and *Anabaena variabilis* (Kim and Lee, 2006). Photosynthesis was significantly decreased when, *Plectonema boryanum* was exposed to 5 $\mu\text{g ml}^{-1}$ of monocrotophos alone or in combination with UV-B treatment (Prasad et al., 2004). Photosynthetic activity of *Nostoc* was stimulated when exposed to 10-1000 μM of dimethoate, 10 μM of butachlor and 2 μM of bensulfuron methyl (Chen et al., 2007).

Photosynthetic activity of *Nostoc calcicola* and *Nostoc muscorum* was severely affected upon exposure to two different concentrations (10 and 20 $\mu\text{g ml}^{-1}$) of endosulfan (Prasad et al., 2011). Photosynthesis of *Anabaena torulosa* was declined when treated with varying concentrations 1.25-5 $\mu\text{g ml}^{-1}$ of anilofos (Singh et al., 2012a). Photosynthesis was inhibited in *Anabaena sphaerica* when treated with malathion dose ranging between 5-30 $\mu\text{g ml}^{-1}$ due to formation of free radicals (Chakraborty et al., 2017). Damage to photosynthetic machinery by the pesticide may have resulted in the generation of reactive oxygen species, which amplified damage to lipids and proteins, leading in oxygen evolution reduction (Chen, 1986; Lohmann and Hedgdorn, 1986; Singh and Tiwari, 1988; Mishra et al., 2008; Sundaram et al., 2011; Raja et al., 2012).

However, in case of oxygen consumption (respiration), the effect of cartap was less pronounced as compared to photosynthesis and concentration dependent decrease was observed. After 1 day of incubation in cartap supplemented media, the cyanobacterium showed 121.87, 98.43, 95.31, 96.87 $\text{nmol } \mu\text{g}^{-1} \text{ Chl } a \text{ min}^{-1}$ oxygen consumption in control, 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively (Fig. 4.11). The oxygen consumption inhibited by 7.68, 10.25, 16.66% in 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively at the end of two days. From the above, it can be concluded that respiratory electron transport chain was inhibited by cartap hydrochloride.

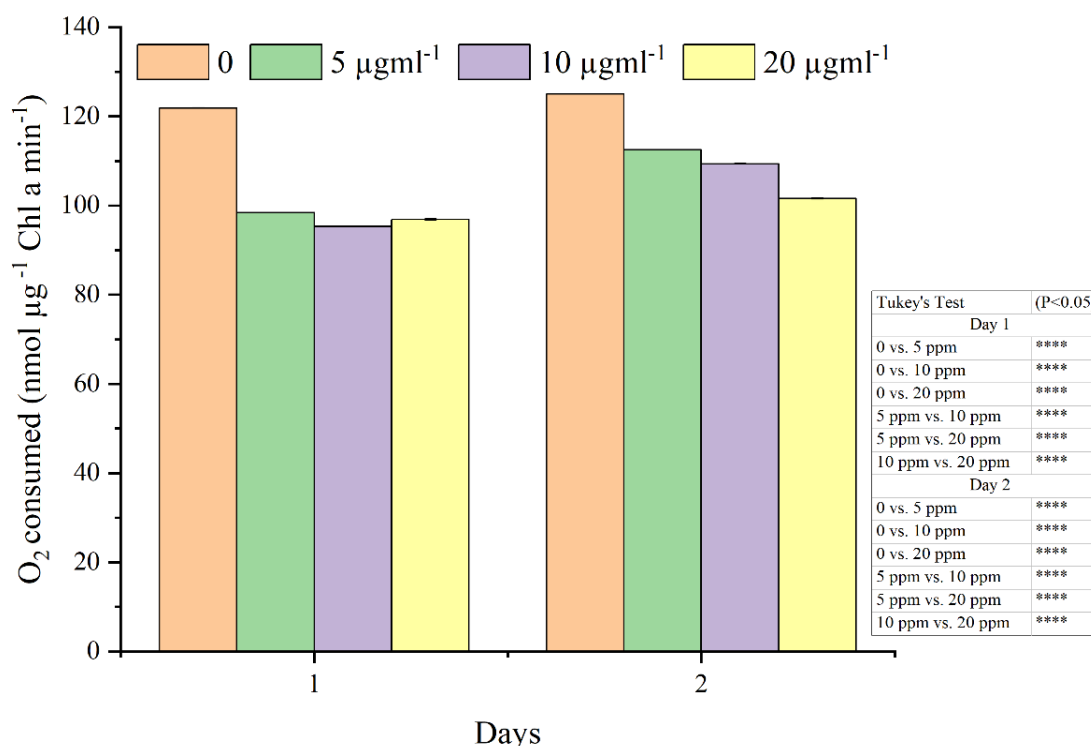


Fig. 4.11: Effect of cartap on respiration of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Cartap affected oxygen consumption (respiration) of *Anabaena variabilis* in same way as photosynthesis. In *Leptolyngbya foveolarum* at higher dose of 40 and 60 $\mu\text{g ml}^{-1}$, oxygen consumption was inhibited by 24 and 45 % but lower concentration of cartap stimulated oxygen consumption by 36% (Singh et al., 2014). Decrease in respiration could be attributable to inhibition of respiratory enzymes. Nimbicidin and monocrotophos affected respiration of *Tolypothrix scytonemoides* in dose dependent manner (Rajendran et al., 2007). Respiratory activity of *Nostoc muscorum* was adversely affected than *Nostoc calcicola* when treated with 5-20 $\mu\text{g ml}^{-1}$ of endosulfan (Prasad et al., 2011). Bensulfuron methyl (2-200 μM) and dimethoate (10-3000 μM) also inhibited respiration in *Nostoc* while dimethoate (10-3000 μM) stimulated the rate of respiration (Chen et al., 2007).

4.1.5 Total nitrogen

The total nitrogen content in cell biomass was estimated to assess the nitrogen contribution by the cyanobacterium by growing it in presence and absence of cartap (Fig. 4.12). Total nitrogen content at the end point of growth was 0.021 in control, which was reduced to 0.017, 0.016 and 0.013%, when grown in presence of 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap corresponding

to a decline by 17.28, 24.29 and 36.44% respectively. This suggests that cyanobacterium is able to contribute some nitrogen through nitrogen fixation even under stress conditions.

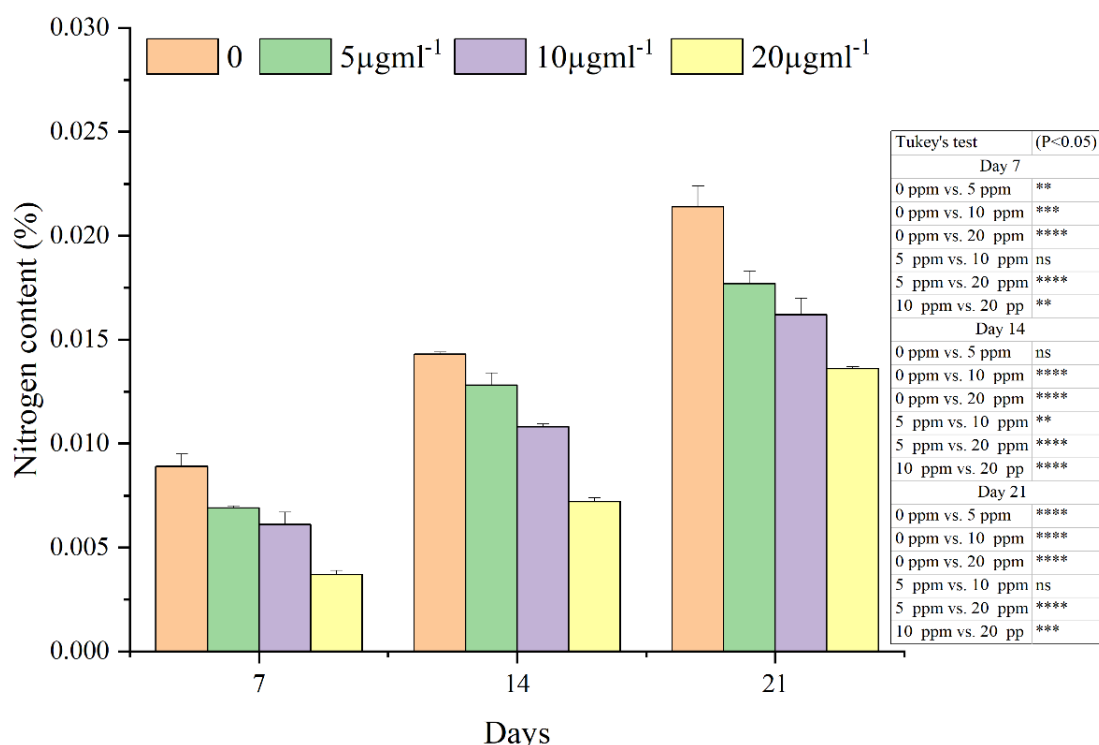


Fig. 4.12: Effect of cartap on nitrogen content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001)

The photo dependent nitrogen fixers in the rice fields include photosynthetic bacteria, free living and symbiotic cyanobacteria. Among these organisms the contribution of photosynthetic bacteria to N-input is low (Singh et al., 2014). Therefore, the nitrogen fixing ability is mainly attributed to free-living cyanobacteria. Cyanobacterial-pesticides interactions have been studied at different stages of rice crop (Dash et al., 2016; Dash et al., 2015). Dose dependent decrease in total nitrogen content in *Anabaena variabilis* ARM 441 correlates well with earlier studies (Dash et al., 2016; Dash et al., 2015; Bueno et al., 2004). Dash et al. (2015) performed a field study to observe the influence of agrochemicals, urea, benthocarb and carbofuran on the biomass and N-yield of native cyanobacteria for three consecutive seasons in water logged rice field. Both urea and benthocarb had deleterious effects, whereas carbofuran promoted growth as well as N-yield, however their combined application was inhibitory. Rogor, endotaf, furadan and sevin at 250 $\mu\text{g ml}^{-1}$ reduced the total

nitrogen content in *Nostoc paludosum* by 88.2, 100, 76.5 and 81.4% respectively (Shinde, 2018).

4.1.6 Effect of cartap on nitrogen uptake

For growth and development, cyanobacteria need nitrate, nitrite, or ammonium as a nitrogen source (Flores and Herrero, 2005). The toxicity of cartap was investigated on *Anabaena variabilis* because its nitrogen metabolism is dependent on photosynthesis.

4.1.6.1 Nitrate uptake

The uptake of nitrate by cyanobacterial cells was studied by growing it in different concentrations ranging from 0.05-5mM (Fig. 4.13). Uptake of nitrate was increased with increase in concentration from 0.2 to 2mM but remained constant upto 5mM. 0.5mM of nitrate was selected for further study to assess cartap effect on cyanobacteria. Uptake studies showed that nitrate uptake was decreased with rising concentration of cartap. After 24 h, the cyanobacterium exhibited 0.31, 0.26, 0.23 μmol nitrate uptake μg^{-1} protein representing inhibition by 8.82, 23.52, 32.35% in 5, 10 and 20 $\mu\text{g ml}^{-1}$. Untreated control cultures exhibited 0.240 to 1.183 μmol nitrate uptake per microgram of protein between 12 to 48 h. In 5, 10 and 20 $\mu\text{g ml}^{-1}$ nitrate uptake ranged between 0.226 to 1.144, 0.150 to 1.033 and .137 to 0.898 from 12 to 48 h representing 3.25, 12.62 and 24.11% decrease at end of 2 days (Fig. 4.14).

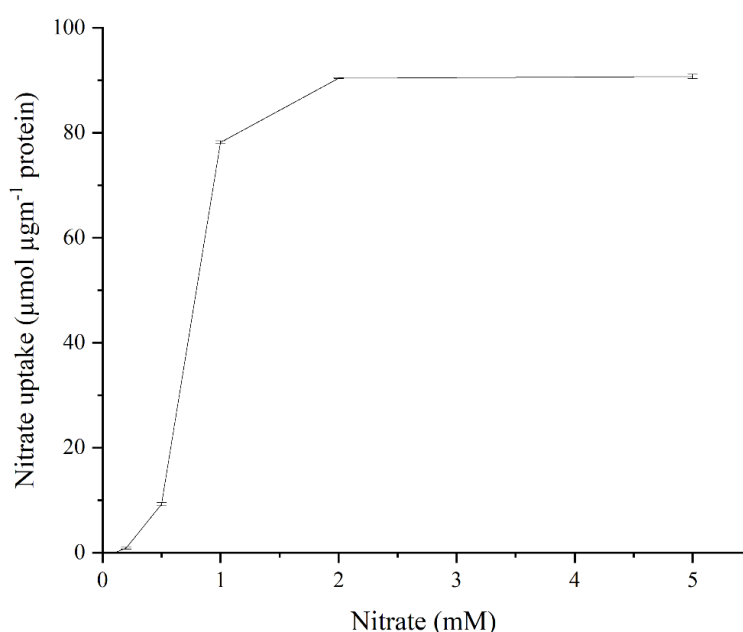


Fig. 4.13: Nitrate uptake by *Anabaena variabilis* after 24 h

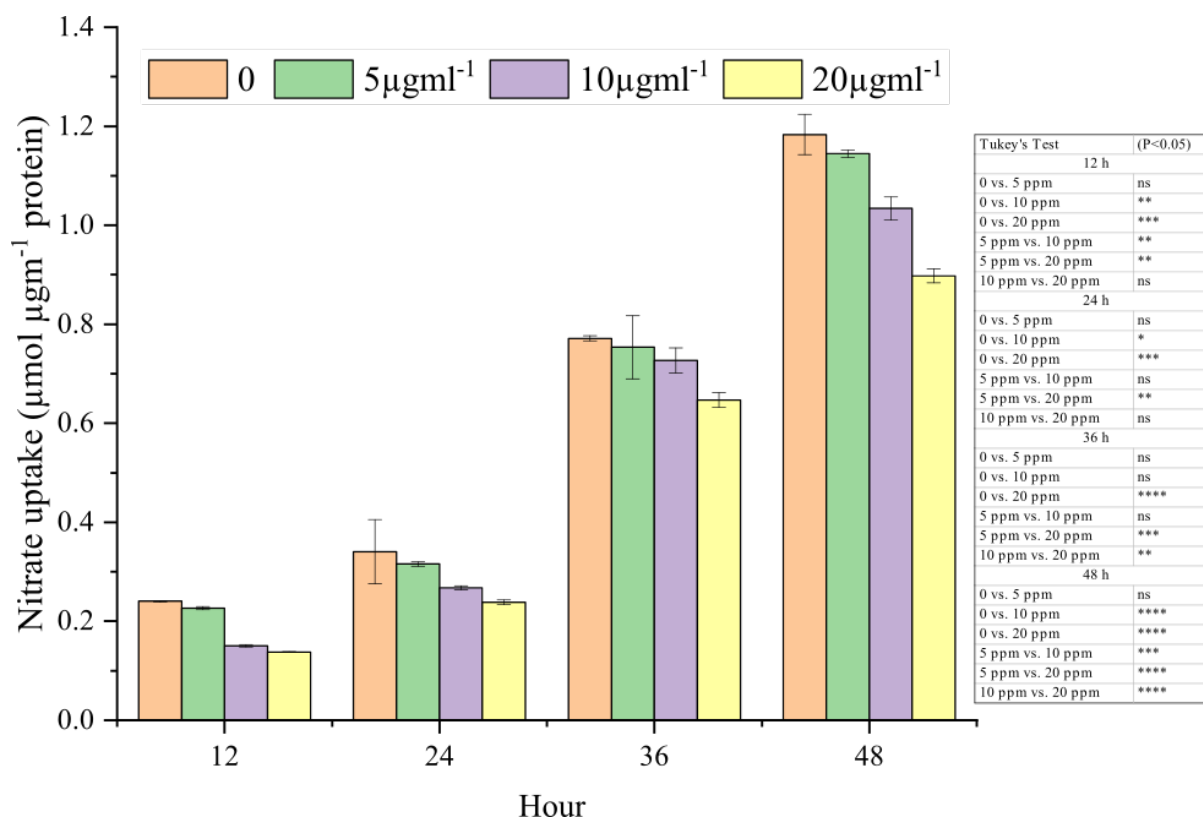


Fig. 4.14: Effect of cartap on nitrate uptake of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

4.1.6.2 Nitrite uptake

The cyanobacterial cells followed the similar pattern of nitrite uptake as nitrate uptake study in varying concentration ranging between 0.05-5mM (Fig.4.15). Increase in nitrite uptake was observed upto 2mM, afterwards it became constant. Therefore, for further study concentration of 0.1mM was selected.

Uptake studies of nitrite revealed that there was marginal effect of cartap on nitrite uptake by cyanobacterium. In control culture, uptake ranged between 0.179 to 0.183 μmol of nitrite μg^{-1} protein from 12 to 48 h. After 12 h, nitrite uptake by cyanobacterium at 5, 10 and 20 $\mu\text{g ml}^{-1}$ cartap treated cells was 0.179, 0.179 and 0.178 $\mu\text{mol} \mu\text{g}^{-1}$ protein respectively (Fig. 4.16). After 24 h, nitrite uptake in 5, 10 and 20 $\mu\text{g ml}^{-1}$ was 0.181, 0.180 and 0.179 μmol nitrite uptake μg^{-1} protein exhibiting 0.04, 0.69 and 1.51% inhibition. The decline in nitrite uptake was negligible which was by 0.111, 0.136 and 0.257% respectively at end of two days of incubation.

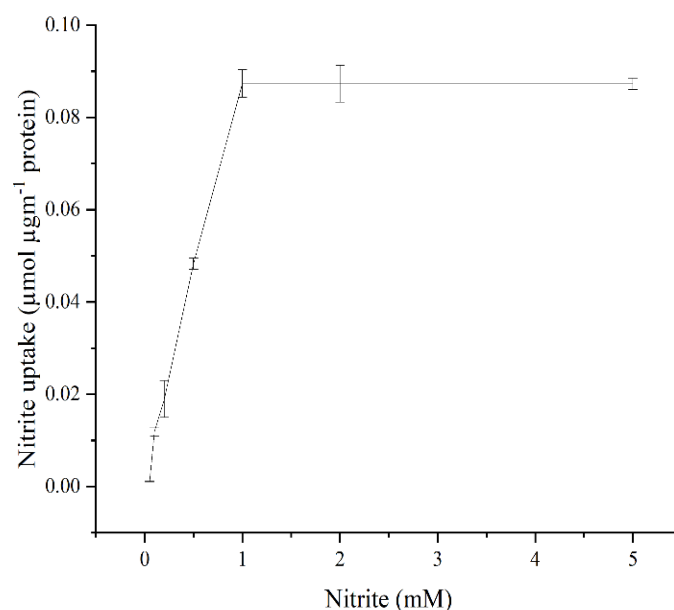


Fig. 4.15: Nitrite uptake by *Anabaena variabilis* after 24 h

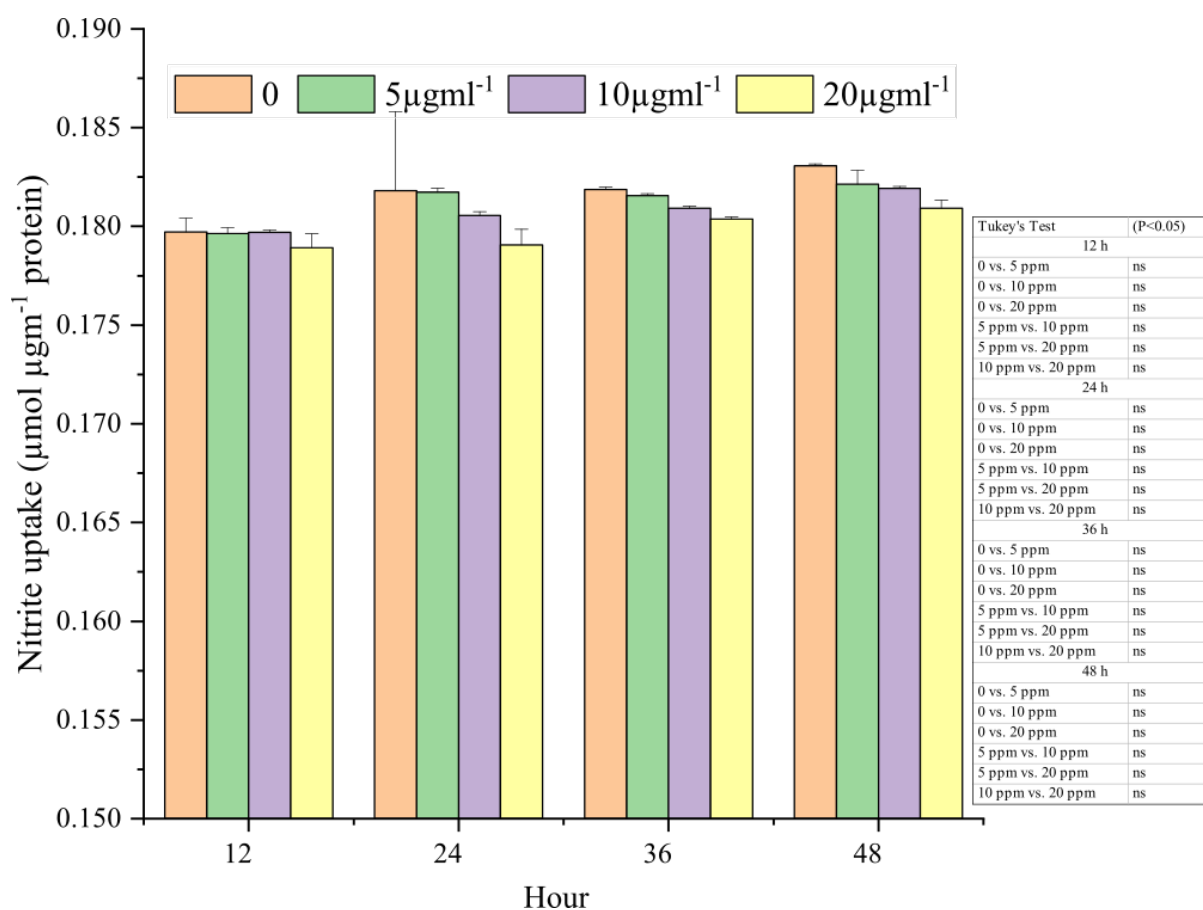


Fig. 4.16: Effect of cartap on nitrite uptake of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

4.1.6.3 Ammonium uptake

The uptake of ammonium by cyanobacterial cells was studied by growing *Anabaena variabilis* at varying concentration (0.05-5mM) of ammonium (Fig. 4.17). The cyanobacterial cells showed increased uptake of ammonium as compared to nitrite and nitrate. Ammonium uptake was increased at 0.05mM to 2mM and thereafter became constant. 0.1mM ammonium was selected for further study.

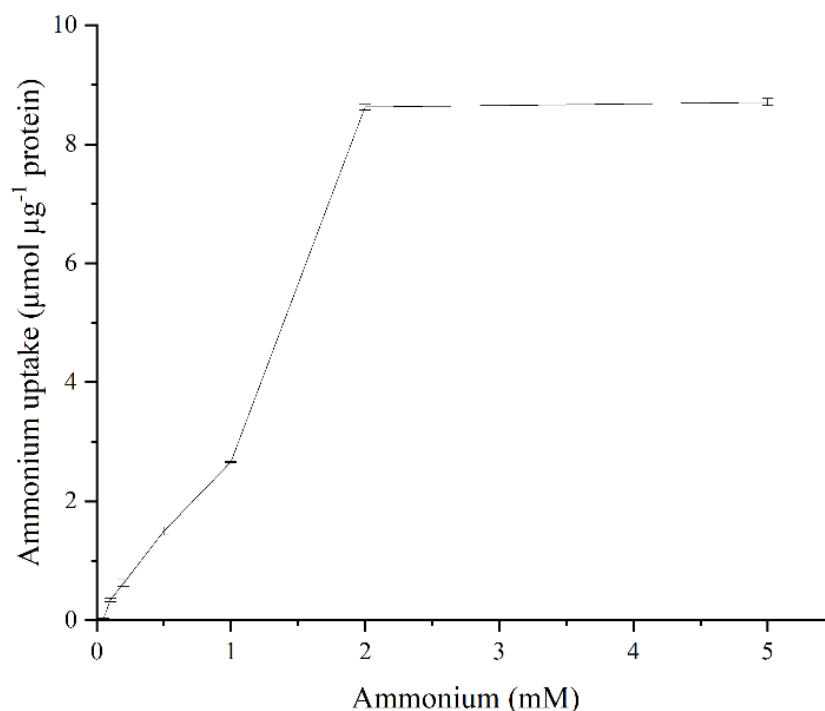


Fig. 4.17: Ammonium uptake by *Anabaena variabilis* after 24 h

Uptake of ammonium in presence of insecticide by cyanobacterium was decreased in concentration dependent manner. Untreated control culture showed ammonium uptake ranging between 0.67 to 0.95 μmol ammonium μg⁻¹ protein from 12 to 48 h. The cyanobacterium showed 0.70, 0.68, 0.62 μmol ammonium uptake μg⁻¹ protein in 5, 10 and 20 μg ml⁻¹ respectively after one day of incubation. After 24 h, 1.40, 4.22, 12.67% decrease in ammonium uptake was observed corresponding to 0.70, 0.68, 0.62 μmol ammonium uptake μg⁻¹ protein. It was further decreased by 15.78, 26.31 and 31.57% after 48 h in 5, 10 and 20 μg ml⁻¹ of cartap treated cells (Fig. 4.18).

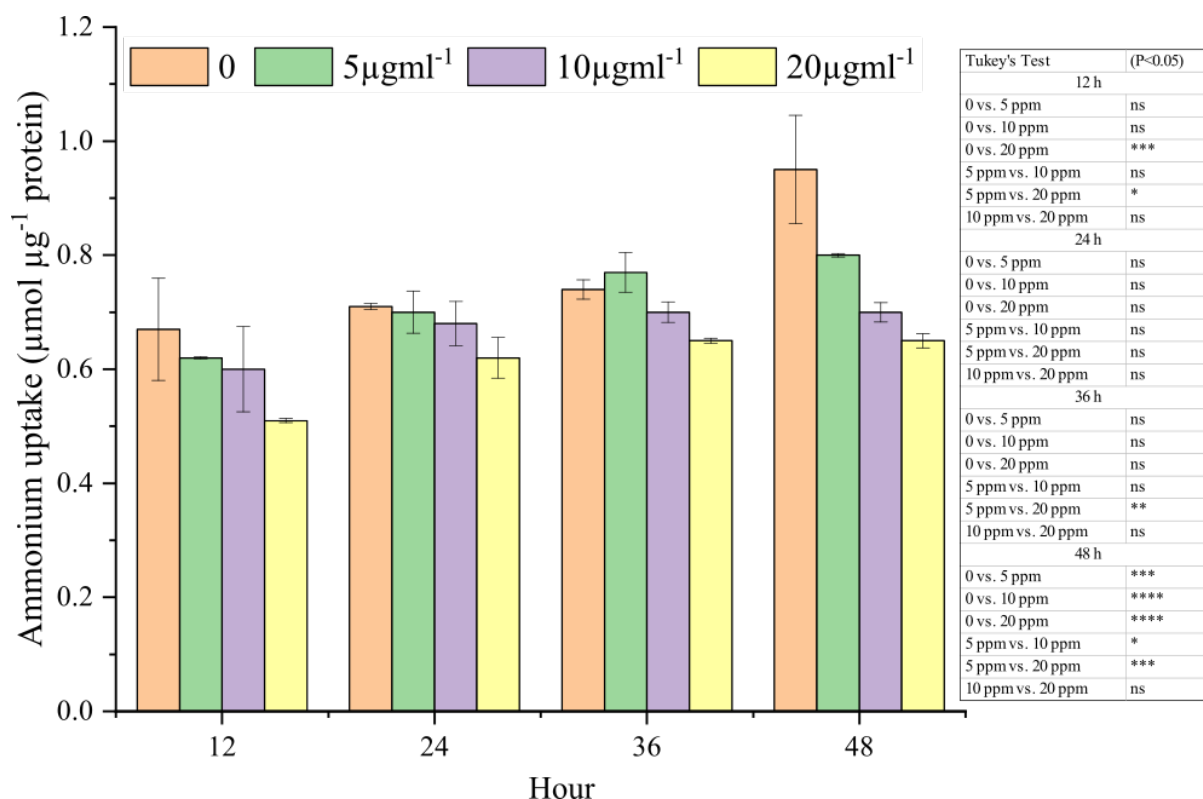


Fig. 4.18: Effect of cartap on ammonium uptake of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Cartap exhibited marginal effect on nitrite uptake whereas nitrate uptake was affected in a dose dependent manner. Overall impact of cartap (0, 5, 10, 20 $\mu\text{g ml}^{-1}$) on nitrate, nitrite and ammonium uptake by cyanobacterium after 24 hour of incubation also suggests that the effect of cartap on the rate of ammonium uptake was the most pronounced of all the nitrogen sources tested, followed by nitrate and nitrite uptake.

In *Leptolyngbya foveolarum*, 13, 61 and 61% inhibition in nitrite uptake was reported at 20, 40 and 60 $\mu\text{g ml}^{-1}$ after 48 h of cartap treatment, while nitrate uptake was inhibited by 15, 23 and 31% respectively (Singh et al., 2014). Inhibition of nitrate uptake was also in *Anabaena PCC 7119* upon trichlorofon treatment (Orus and Marco 1993). In *Nostoc sp.*, *Anabaena doliolum* and *Nostoc calcicola*, decline in ammonium and nitrate uptake was reported when treated with Saturn and Machete. Low concentration of 2, 4-D had no effect on ammonium uptake whereas nitrate uptake was increased. However, at higher doses uptake of both nitrate and ammonium was inhibited (Mishra and Pandey, 1989).

26 and 12 % reduction was observed in ammonium and nitrate uptake respectively, when *Anabaena torulosa* was treated with 5 $\mu\text{g ml}^{-1}$ of anilofos (Singh et al., 2012a). Nitrate uptake was decreased by 14-39% in *Phormidium foveolarum* and 54-59% *Nostoc muscorum* upon treatment with 10-20 $\mu\text{g ml}^{-1}$ of oxyfluorfen (Sheeba et al., 2011). In *Anabaena variabilis*, treatment with butachlor, dimethoate, cypermethrin and isoproturon resulted in decline in nitrate and nitrite uptake in a time dependent manner (Aftab et al., 2013).

Uptake of ammonium in cyanobacterium was shown to be a membrane potential driven transport process (Montesinos et al., 1998). It is possible that cartap interfered with the membrane potential of the cell membrane thus decreasing its permeability, allowing slow ammonium uptake.

4.1.6 Effect of cartap on nitrogen assimilating enzymes

In cyanobacteria, nitrate and nitrite assimilation takes place in two steps: nitrate and nitrite uptake by the cells, followed by reduction to ammonium (Avissar, 1985; Herrero et al., 1981, 1991; Flores and Herrero, 2005). The enzymes nitrate reductase (NR) and nitrite reductase (NiR) catalyze nitrate reduction in cyanobacteria in two consecutive steps. Nitrate is transformed to nitrite in the cells by NR, which is then reduced to ammonium by NiR. The nitrite reductase enzyme reduces both the forms of nitrite (nitrite formed within cells or nitrite directly taken up by cells) into ammonium. So, cartap toxicity was assessed on enzyme nitrate reductase and nitrite reductase. NR and NiR activities were affected in both dose and time dependent manner in *Anabaena variabilis*.

4.1.7.1 Nitrate reductase (NR) and Nitrite reductase (NiR)

Nitrate reductase activity of *Anabaena variabilis* was measured at specific time interval of 24, 48 and 72 h. The activity of nitrate reductase enzyme was inhibited with increasing concentration of cartap. The control culture showed NR activity of 1.38 nmol nitrite formed mg^{-1} protein min^{-1} after 72 h. The cyanobacterium exhibited NR activity of 1.09, 0.69 and 0.10 nmol nitrite formed mg^{-1} protein min^{-1} in 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively after 24 h of incubation. After 48 h, the NR activity at 5, 10 and 20 $\mu\text{g ml}^{-1}$ was 1.29, 0.99, 0.59 nmol nitrite formed mg^{-1} protein min^{-1} corresponding to inhibition percentage of 6.5, 28.26, and 57.24% respectively. The cyanobacterium exhibited NR activity of 1.29, 1.09 and 0.69

nmol nitrite formed mg^{-1} protein min^{-1} in 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively after 72 h of incubation. (Fig. 4.19).

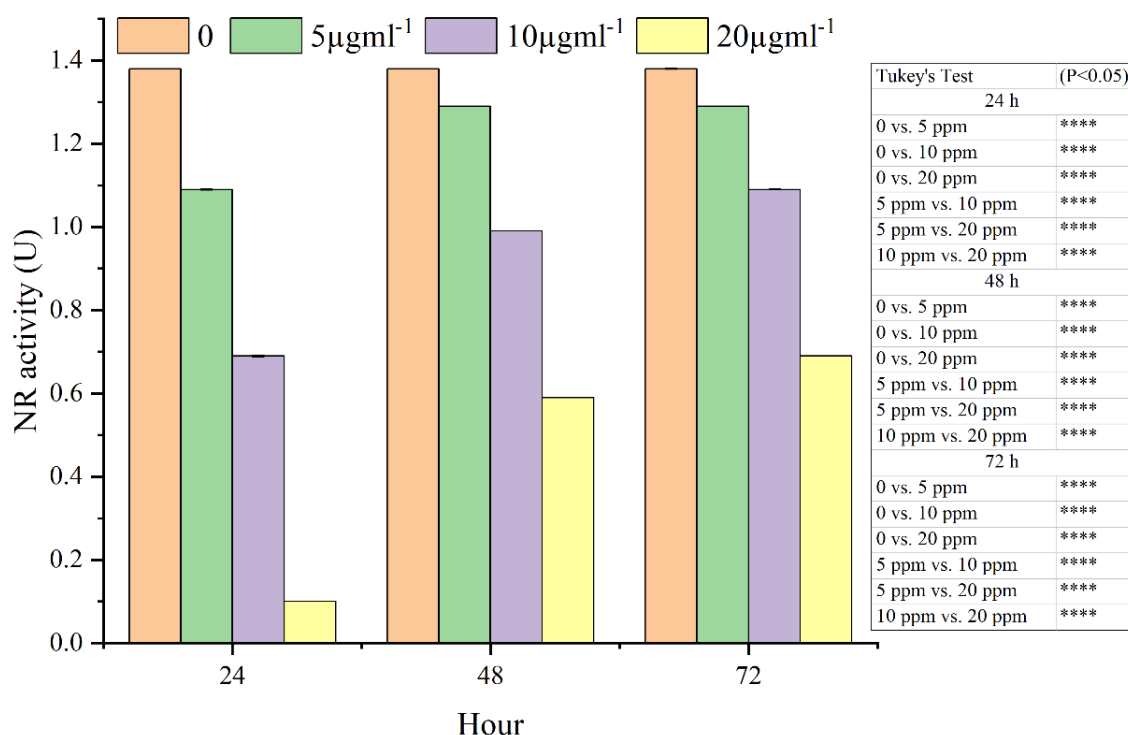


Fig. 4.19: Effect of cartap on nitrate reductase (NR) of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The activity of nitrite reductase was suppressed in same way as nitrate reductase in presence of cartap. In control culture, NiR activity was increased from 2.85 nmol nitrite consumed mg^{-1} protein min^{-1} to 3.54 nmol nitrite consumed mg^{-1} protein min^{-1} from 24 to 72 h. After 24 h, the activity in treated cultures was 1.97, 1.34, 0.664 nmol nitrite consumed mg^{-1} protein min^{-1} in 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively. The cyanobacterium exhibited NiR activity of 2.52, 2.37 and 0.716 nmol nitrite consumed mg^{-1} protein min^{-1} in 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively after 48 h of incubation. At the end of 72 h the treated cells exhibited NiR activity 3.19, 2.37 and 0.970 nmol nitrite consumed mg^{-1} protein min^{-1} in 5, 10 and 20 $\mu\text{g ml}^{-1}$ representing inhibition percentage of 9.88, 33.05 and 72.58 % respectively (Fig. 4.20).

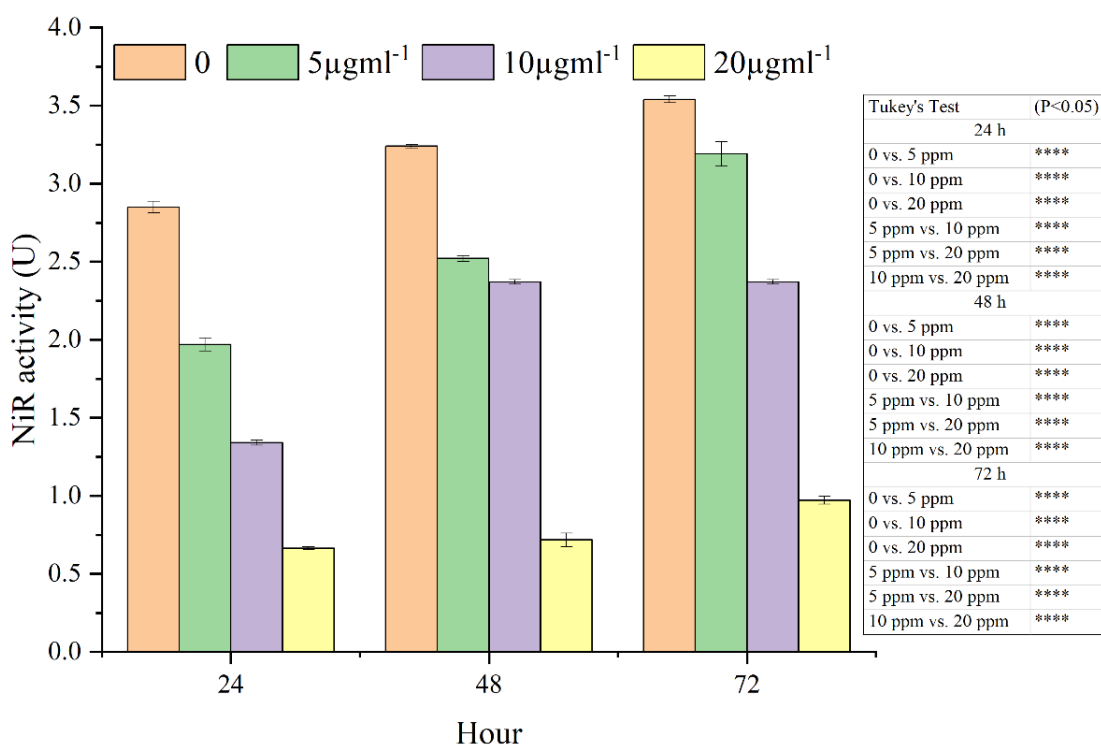


Fig. 4.20: Effect of cartap on nitrite reductase (NiR) of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

At 20 $\mu\text{g ml}^{-1}$ of cartap, the activity of both the enzymes were decreased. Low levels of enzyme activity could be attributable to low uptake of nitrate and nitrite in presence of cartap.

In *Leptolyngbya. foveolarum*, there was slight effect on the activity of NR and NiR but at higher dose (60 $\mu\text{g ml}^{-1}$) NR and NiR activity was inhibited by 48.2 and 36.5% respectively in presence of cartap hydrochloride (Singh et al., 2014). In a report NR activity of *Westiellopsis prolifica* and *Anabaena sp.* were inhibited by sevin (Adhikary et al., 1984). Nitrate reductase activity in *Nostoc sp.* was inhibited at 5-50 $\mu\text{g ml}^{-1}$ of carbaryl, whereas at 3-40 $\mu\text{g ml}^{-1}$ of endosulfan, 15-120 $\mu\text{g ml}^{-1}$ of 2, 4-D, 7.5-60 $\mu\text{g ml}^{-1}$ of tebuconazole and 15-200 $\mu\text{g ml}^{-1}$ of pencycuron nitrate reductase activity was inhibited in *Westiellopsis prolifica*, *Anabaena Jertilissima* and *Aulosira fertilissima* (Bhunja et al., 1994; Kumar et al., 2010a, 2012).

Interference of 20 and 50 $\mu\text{g ml}^{-1}$ of carbofuran with photosynthetic machinery of *Nostoc linckia*, *Nostoc muscorum* and *Chlorella vulgaris* resulted in decreased NR activity

(Megharaj et al., 1993). Combination of two different concentrations of oxyfluorfen (10 and 20 $\mu\text{g ml}^{-1}$) and UV-B sharply decreased nitrate reductase in *Nostoc muscorum* but same treatment resulted in increased NR activity in *Phormidium foveolarum* (Sheeba et al., 2011). NR activity was decreased by 34-66% upon treatment with 10 μM each of butachlor, dimethoate, cypermethrin and isoproturon (Aftab et al., 2013). 7-60 $\mu\text{g ml}^{-1}$ of tebuconazole inhibited the activity of nitrate reductase by 90-93% in *Westiellopsis prolifica*, *Aulosira fertilissima* and *Anabaena fertilissima*, while 3-60 $\mu\text{g ml}^{-1}$ of endosulfan reduced activity of this enzyme between 77-95% in these cyanobacteria (Kumar et al., 2012).

4.1.7.2 Glutamine synthetase (GS) activity

Impact of cartap on glutamine synthetase activity of *Anabaena variabilis* is shown in Fig. 4.21. Control culture exhibited glutamine synthetase activity of 2.01, 2.35 and 2.43 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} in presence of cartap after 24, 48 and 72 h respectively. GS activity was decreased as the concentration of cartap was increased in culture medium. After 24 h, the inhibition percentage in GS activity was 2.51, 13.57 and 77.73% corresponding to GS activity of 1.95, 1.73, 0.44 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap.

The cyanobacterium exhibited GS activity of 2.14, 2.10 and 1.35 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} in presence of 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively after 48 h. The GS activity was 2.22 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} in presence of 5 $\mu\text{g ml}^{-1}$ of cartap, 2.15 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} in 10 $\mu\text{g ml}^{-1}$ and 1.58 nmol γ -glutamate hydroxamate formed mg^{-1} protein min^{-1} in 20 $\mu\text{g ml}^{-1}$ after 72 h of incubation.

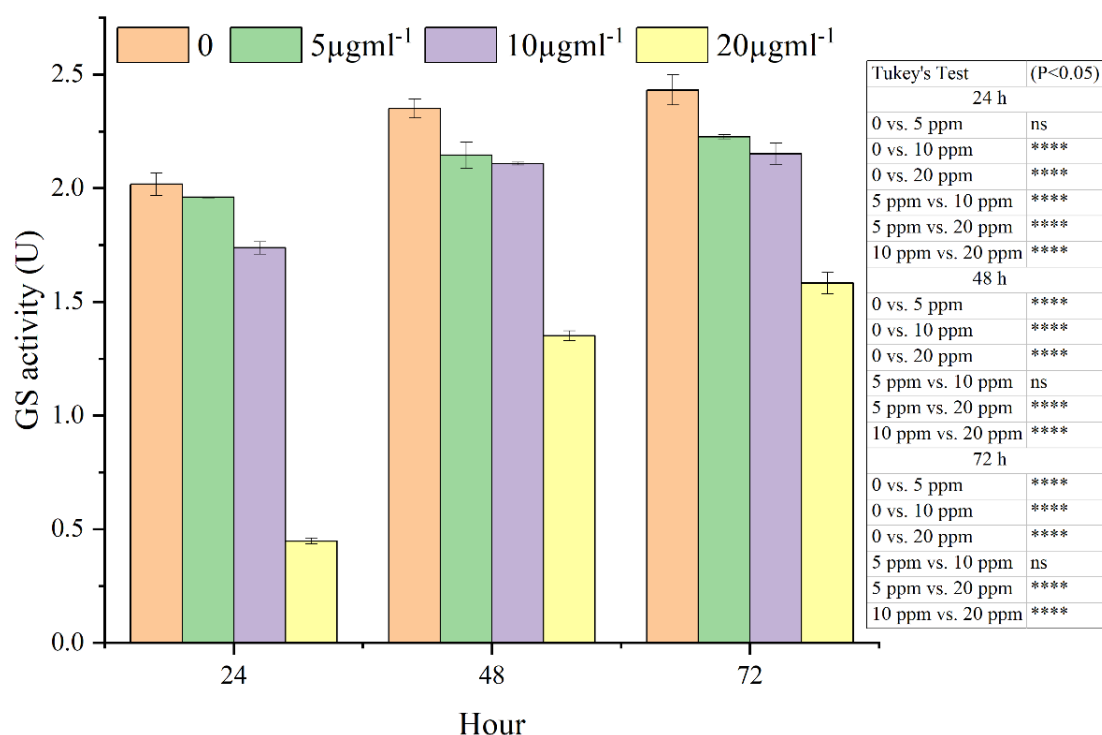


Fig. 4.21: Effect of cartap on glutamine synthetase of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

In cyanobacteria, ammonium generated by nitrate and nitrite reductase or by direct absorption is integrated into the carbon skeleton *via* the action of glutamine synthetase (GS) by using the GS-GOGAT system (Flores and Herrero, 2005). In *Anabaena variabilis*, GS activity was decreased in a dose dependent manner after 48 h showing decline by 8.76, 10.34 and 42.54 % at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively. This decrease can be correlated with reduced nitrite and nitrate uptake rather than direct effect of cartap on activity of glutamine synthetase.

GS activity of *Leptolyngbya foveolarum* showed 37.5, 50.3 and 50.3% decrease when treated with 20, 40 and 60 $\mu\text{g ml}^{-1}$ cartap, after 24 h (Singh et al., 2014). Carbofuran (0.25 $\mu\text{g ml}^{-1}$ and 0.5 $\mu\text{g ml}^{-1}$) significantly increased the glutamine synthetase activity of *Anabaena sphaerica*, *Westiellopsis prolifica* and *Nostoc hatei*, but endosulfan and chlorpyrifos treatment showed inhibition it (Jha and Mishra, 2005). In *Tolypothrix scytonemoides*, decline in GS activity was observed upon exposure to nimbecidin, bavistin and monocrotophos (Rajendran et al., 2007). 9-22% reduction in GS activity was observed after treatment of *Anabaena torulosa* with 1.25-5 $\mu\text{g ml}^{-1}$ of anilofos (Singh et al., 2012a). *Aulosira fertilissima*

when treated with 3-12 $\mu\text{g ml}^{-1}$ of endosulfan decreased GS activity by 61-71% (Kumar et al., 2009) but 2, 4 D exposure to same cyanobacterium resulted in decline of GS activity by 72-95% (Kumar et al., 2010a). Treatment of tebuconazole and endosulfan resulted in inhibition of GS activity by 59-95% and 77-97% respectively in *Aulosira fertilissima*, *Westiellopsis prolifica* and *Anabaena fertilissima* (Kumar et al., 2012). The effect of cartap was strongest on NiR activity, followed by NR activity, and GS activity, among these three enzymes.

4.1.8 Enzymatic antioxidants

Insecticide toxicity leads to the formation of free radicals and cyanobacteria respond to this stress by activating antioxidant enzymatic defense system. The key enzyme of antioxidant defense system is super oxide dismutase (SOD) which neutralizes singlet oxygen and ROS such as superoxide radicals leading to generation of the less toxic H_2O_2 , which is still dangerous for cells (Lesser, 2006) and requires removal either by peroxidase (POD) or by catalase (CAT) enzyme. Another enzyme involved in the detoxification of reactive oxygen species (ROS) is glutathione-S-transferase (GST). We were interested in investigating strategies adopted by cyanobacteria to circumvent the toxic effect of the cartap. Therefore, activities of four different anti-oxidant enzymes, SOD, CAT, POD and GST were measured. The effect of cartap on different antioxidant defense system enzymes in *Anabaena variabilis* ARM 441 was studied at a regular interval of 2 days upto 6 days.

4.1.8.1 Superoxide dismutase

Superoxide dismutase activity of *Anabaena variabilis* was significantly increased when treated with 5-20 $\mu\text{g ml}^{-1}$ of cartap. The 20 $\mu\text{g ml}^{-1}$ cartap treated culture showed maximum activity of enzyme followed by 10 and 5 $\mu\text{g ml}^{-1}$. In 20 $\mu\text{g ml}^{-1}$, catalase activity was increased from 0.74 U on day 2 to 5.42 U on day 6. After 6 days, the catalase activity in 5 and 10 $\mu\text{g ml}^{-1}$ was 4.20 and 4.65 U respectively. The cyanobacterium showed 69.05, 108.57 and 129.05% increase in SOD activity in 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap, respectively, after 4 days with respect to control without insecticide (Fig. 4.22).

Algae contain several enzymatic and non-enzymatic antioxidant defense systems to maintain the concentration of ROS to protect cells from damage. Cartap stimulated the SOD activity by 180, 154 and 131% in 20, 40 and 60 $\mu\text{g ml}^{-1}$ in cartap treated cyanobacterial cells of *Leptolyngbya foveolarum* (Singh et al., 2014). SOD activity was increased in dose dependent

manner in *Nostoc* when treated with different concentrations of butachlor varying between 20-80 $\mu\text{g ml}^{-1}$ (He et al., 2013).

In *Plectonema boryanum*, three different concentrations of endosulfan (5, 10, 20 $\mu\text{g ml}^{-1}$) stimulated the SOD activity by 23, 31 and 58% (Prasad et al., 2005). SOD activity was induced in *Anabaena variabilis*, *Nostoc muscorum* and *Aulosira fertilissima* due to endosulfan treatment (Kumar et al., 2008a). *Chlorella vulgaris* when treated with paraquat and glufosinate (0.5 and 0.75 μM), there was increase in SOD activity by 3-4 times (Qian et al., 2008, 2009).

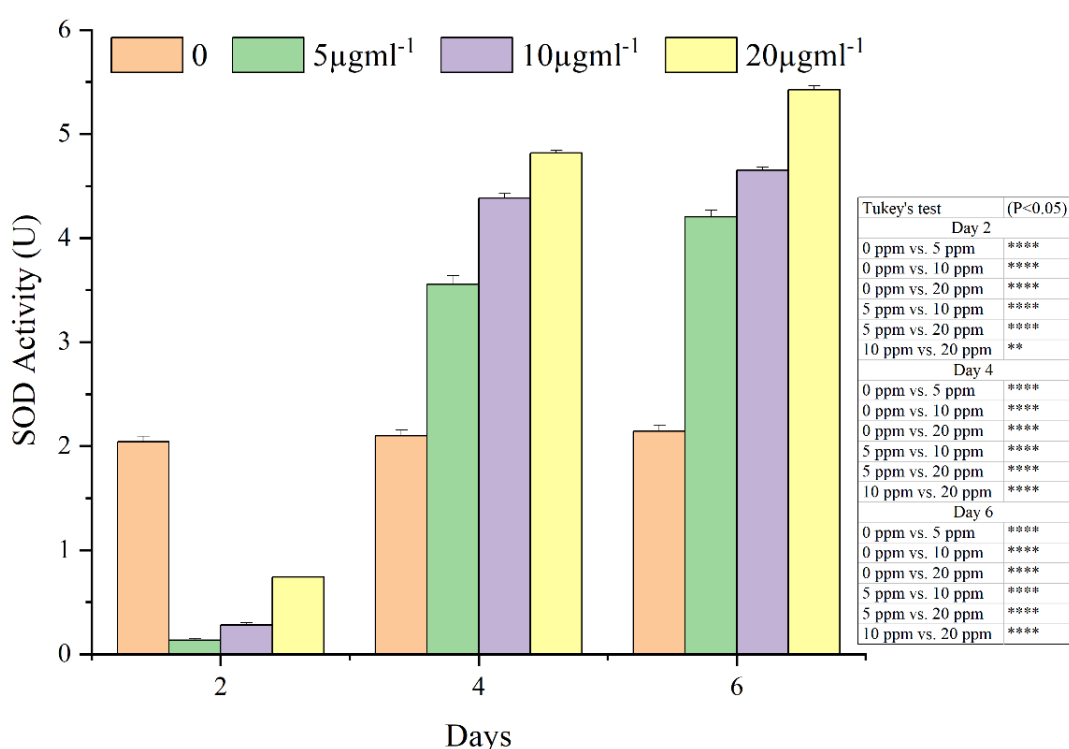


Fig. 4.22: Effect of cartap on superoxide dismutase (SOD) activity of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Phormidium valderianum exposure to chlorpyrifos and *Anabaena cylindrica* exposure to bentazon resulted in increased activity of enzyme in dose dependent manner (Palanisami et al., 2009; Galhano et al., 2010). SOD activity of *Calothrix brevissima* was increased by 79% when exposed to carbaryl (40 $\mu\text{g ml}^{-1}$) (Habib et al., 2011).

Galhano et al. (2011b) reported that molinate stress (0.75-2mM) caused increased SOD activity by 34-92% in *Nostoc muscorum*. SOD activity was significantly increased in *Synechocystis Sp. Strain PUPCCC 64*, *Oscillatoria simplicissima* and *Anabaena torulosa* after anilofos exposure (Singh and Sandhu, 2010; Singh et al., 2012a, 2013). SOD activity of *Fisherella sp.* was increased by 43.8 % after 8 days of treatment with methyl parathion (Tiwari et al., 2018). Malathion exposure increased SOD activity by 1.73 and 2.31 fold in 20 and 30 $\mu\text{g ml}^{-1}$ respectively in *Anabaena sphaerica* (Chakraborty et al., 2017).

4.1.8.2 Peroxidase

Anabaena variabilis showed increased levels of peroxidase after cartap exposure. Peroxidase activity was maximum in 20 $\mu\text{g ml}^{-1}$ followed by 10 $\mu\text{g ml}^{-1}$ and 5 $\mu\text{g ml}^{-1}$. The cyanobacterium showed increase in POD activity by 62.5, 187.5 and 287.5% after 2 days of cartap treatment (Fig. 4.23) at 5, 10 and 20 $\mu\text{g ml}^{-1}$ cartap respectively. In 20 $\mu\text{g ml}^{-1}$, POD activity increased from 0.31U on day 2 to 0.14 U on day 6. In 5 and 10 $\mu\text{g ml}^{-1}$, POD activity increased to .09 and 0.13 U after 6 days of treatment.

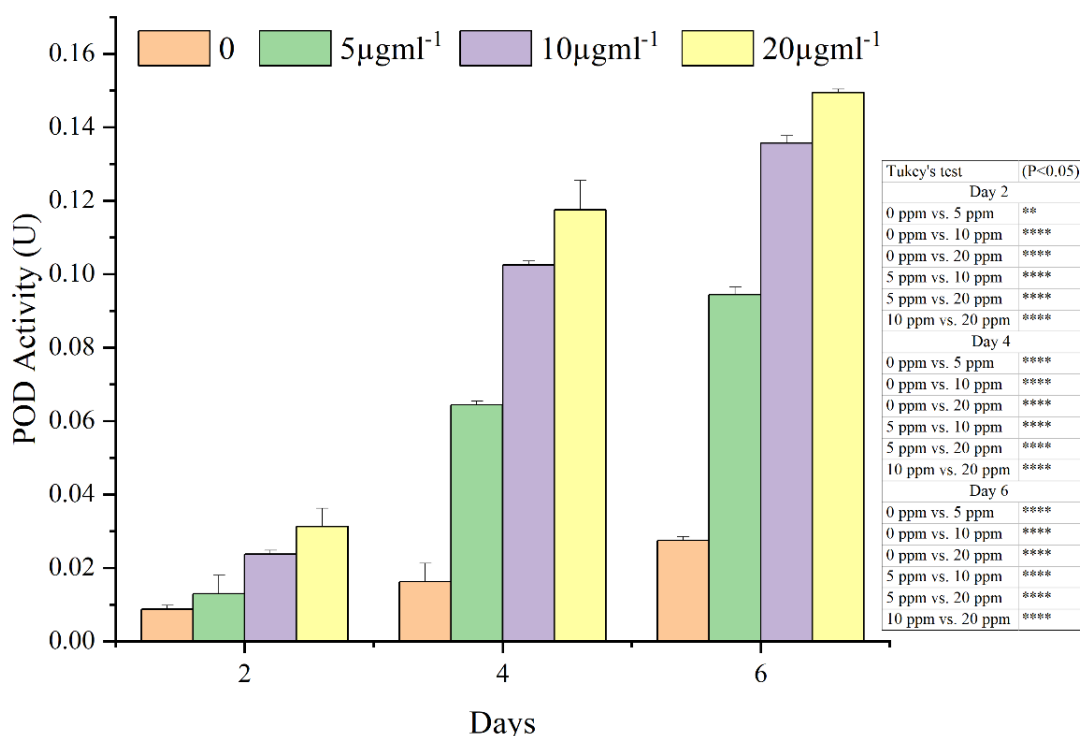


Fig. 4.23: Effect of cartap on peroxide (POD) activity of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Butachlor at low dose ($40\mu\text{g ml}^{-1}$) stimulated the activity of POD as compared to high dose of $80\mu\text{g ml}^{-1}$ in rice field cyanobacterium (He et al., 2013). In *Plectonema boryanum*, POD activity was increased by 20, 30 and 50% when treated with three different doses 5, 10 and $15\mu\text{g ml}^{-1}$ of endosulfan (Prasad et al., 2005). *Phormidium valderianum* exposure to chlorpyrifos ($40\mu\text{g ml}^{-1}$) resulted in stimulated POD activity (Palanisami et al., 2009). Treatment with molinate (0.75-2.0 mM) resulted in decreased activity in *Nostoc muscorum* by 70-88% (Galhano et al., 2011b). 79-191 % increase in activity of peroxidase enzyme was observed in *Anabaena variabilis* after malathion treatment ($25\text{-}100\mu\text{g ml}^{-1}$) (Manikar et al., 2013). *Anabaena torulosa*, *Synechocystis sp. strain* PUPCCC 64 and *Oscillatoria simplicissima* showed stimulated activities after anilofos exposure (Singh and Sandhu, 2010; Singh et al., 2012a, 2013).

4.1.8.3 Catalase

Catalase activity of *Anabaena variabilis* was significantly increased when treated with $5\text{-}20\mu\text{g ml}^{-1}$ of cartap. The $20\mu\text{g ml}^{-1}$ cartap treated culture showed maximum activity of enzyme followed by 10 and $5\mu\text{g ml}^{-1}$. In $20\mu\text{g ml}^{-1}$, catalase activity was increased from 0.342 U on day 2 to 0.918 U on day 6. After 6 days, the catalase activity in 5 and $10\mu\text{g ml}^{-1}$ was 0.244 and 0.576 U respectively. The cyanobacterium showed increase in catalase activity by 8.33, 57.40 and 216.66% (Fig. 4.24) in 5, 10 and $20\mu\text{g ml}^{-1}$ cartap respectively.

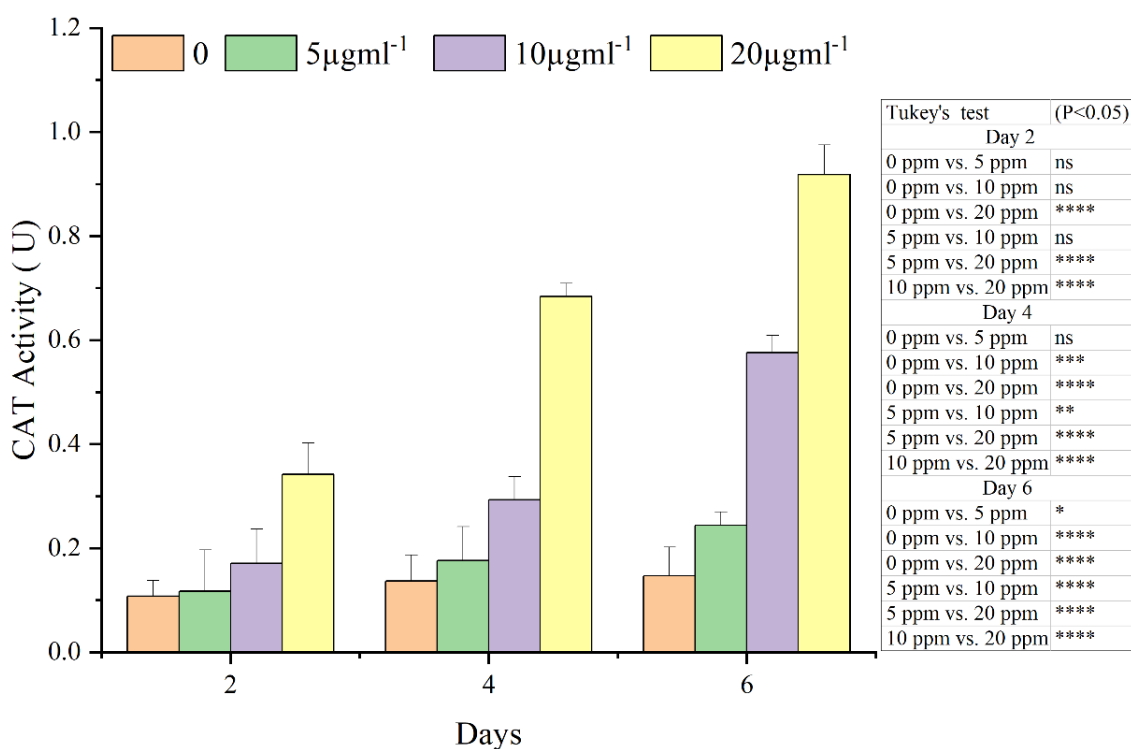


Fig. 4.24: Effect of cartap on catalase (CAT) activity of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Paraquat exposure (0.5 and 0.75 μ M) to *Chlorella vulgaris* for 1 day resulted in increase in enzyme activity by 3.0 and 6.4 times respectively (Qian et al., 2009). In *Nostoc muscorum* molinate stress (0.75-2.0 mM) for 72 h resulted in decrease in catalase activity by 34-92% (Galhano et al., 2011b). Treatment with oxyfluorfen (10 μ g ml⁻¹) stimulated the catalase activity of *Nostoc muscorum* but inhibited the activity in *Phormidium foveolarum* (Sheeba et al., 2011).

Malathion exposure (25-100 μ g ml⁻¹) resulted in stimulation of catalase activity by 16-156% in *Anabaena variabilis* (Manikar et al., 2013). Catalase activity was significantly increased when *Anabaena torulosa*, *Synechocystis sp. strain sp.* PUPCCC 64 and *Oscillatoria simplicissima* was exposed to anilofos (Singh and Sandhu, 2010; Singh et al., 2012a, 2013). In *Nostoc sp.*, catalase was stimulated by 1.08 fold upon exposure to 80 μ g ml⁻¹ of butachlor (He et al., 2013). Malathion exposure (20 and 30 μ g ml⁻¹) increased catalase activity of *Anabaena sphaerica* by 1.81 and 2.16 fold respectively (Chakraborty et al., 2017).

4.1.8.4 Glutathione S transferase (GST) activity

The activity of enzyme Glutathione S transferase was stimulated when cyanobacterium was under cartap stress. In control culture, the GST activity was 4.68 nmol min⁻¹ mg⁻¹ protein at the end of 6 days. In cartap treated cyanobacterial culture, GST activity increased from 6.25 to 14.06 nmol min⁻¹ mg⁻¹ protein at 5 µg ml⁻¹, 10.93 to 15.62 nmol min⁻¹ mg⁻¹ protein at 10 µg ml⁻¹ and 15.63 to 29.68 nmol min⁻¹ mg⁻¹ protein at 20 µg ml⁻¹ of cartap from day 2 to day 6 (Fig. 4.25). The cyanobacterium showed 33.26, 133.04, 233.04 % increase at 5, 10 and 20 µg ml⁻¹ in GST activity after 2 days of incubation.

The activity of enzyme Glutathione-S-transferase (GST) of *Anabaena variabilis* was significantly affected in 20 µg ml⁻¹ than 5 and 10 µg ml⁻¹ when exposed to cartap for 6 days. The GST activity in 20 µg ml⁻¹ was increased from 15.62 U on day 2 to 29.68 U on day 6. In 5 and 10 µg ml⁻¹ of cartap treatment, the GST activity was 15.62 and 29.68 U respectively.

Scenedesmus obliquus showed stimulated GST activity by 136 and 120% when treated with 10 and 0.015 µg ml⁻¹ of diuron (Geoffroy et al., 2002). GST activity was dramatically reduced in dose and time dependent manner in *Nostoc muscorum* upon exposure to molinate (0.75-2.0 mM) (Galhano et al., 2011b). Three cyanobacterial strains exposed to butachlor resulted in stimulated GST activity in *Anabaena* L31, *Anabaena doliolum* and *Anabaena* 7120 by 2.49, 1.92 and 2.1 fold after 7 days (Agrawal et al., 2014).

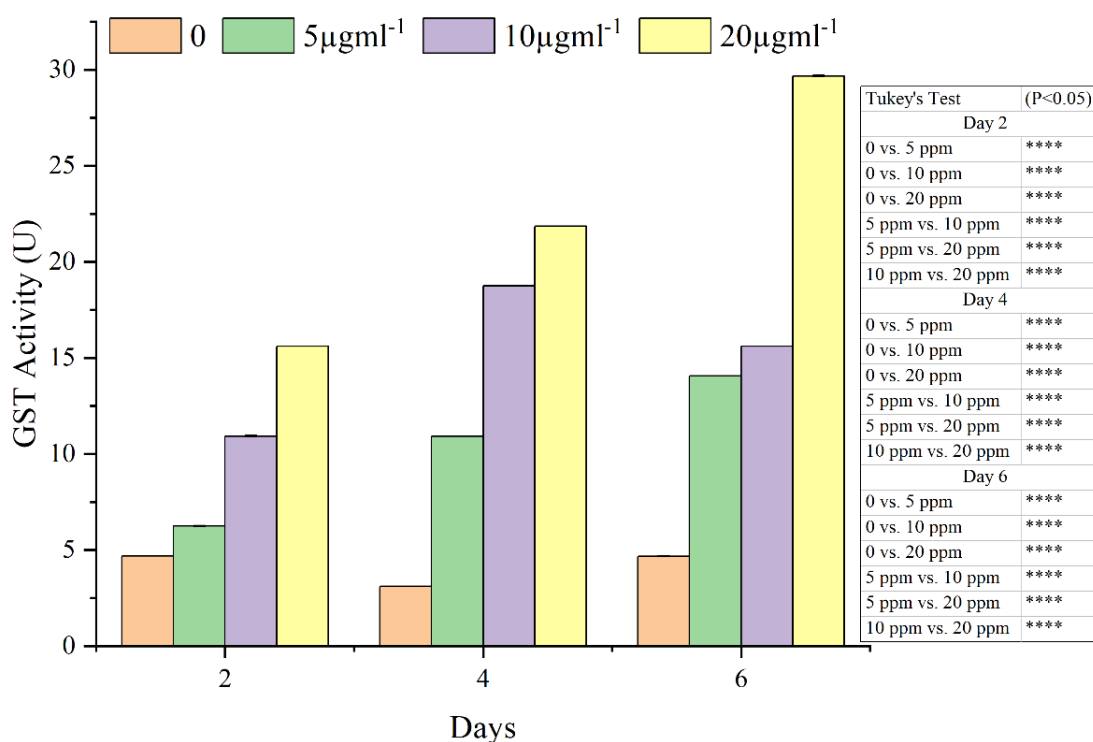


Fig. 4.25: Effect of cartap on Glutathione S transferase (GST) activity of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

The activities of POD, SOD, CAT and GST were increased in a dose dependent manner. Interestingly increased levels of all of the enzymes were observed suggesting that cyanobacteria accumulate more antioxidants, so as to detoxify the various free radicals generated. The findings suggested that these enzymes are helpful in mitigating the oxidative stress caused by cartap.

4.1.9 Non enzymatic antioxidants

To combat insecticide stress, low molecular weight compounds like glutathione (GSH) and ascorbate are synthesized in plants and microorganisms.

4.1.9.1 Glutathione

Glutathione content of *Anabaena variabilis* was significantly affected upon cartap exposure. Effect of cartap on glutathione content was studied for 8 days at an interval of 2 days in an exponentially growing culture. Glutathione was significantly increased by 150, 128.57 and

69.04% at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap, respectively on 8th day (Fig. 4.26). In *Anabaena variabilis* glutathione content decreased with increase in cartap dose.

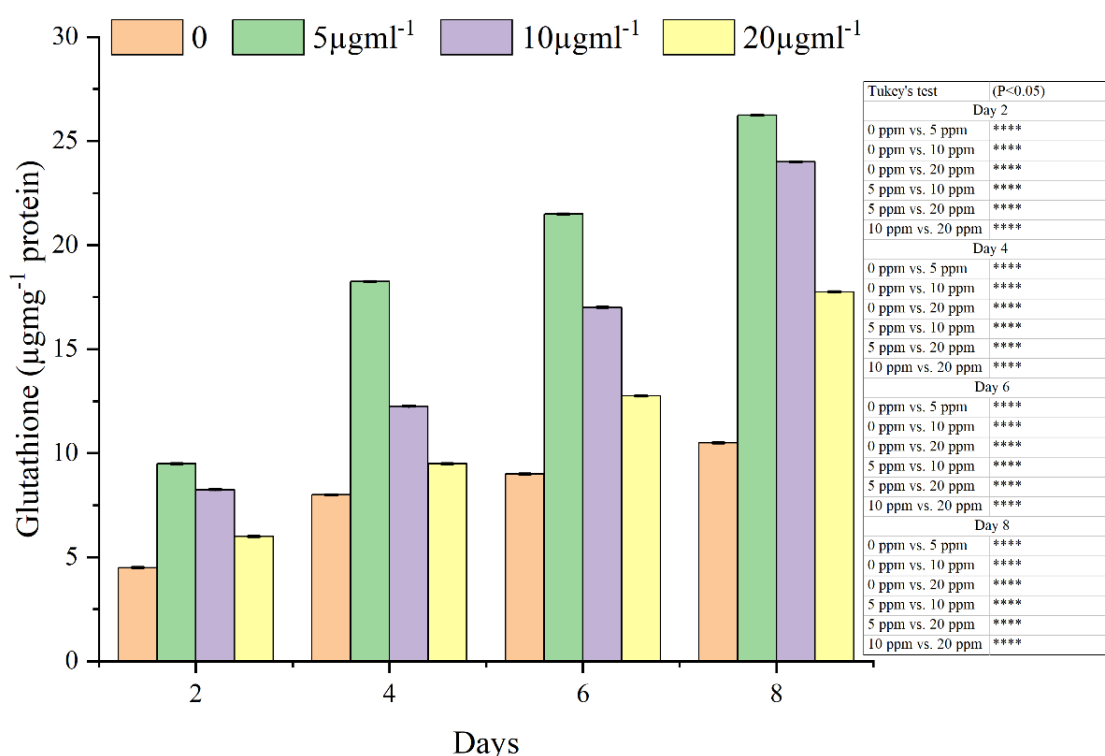


Fig. 4.26: Analysis of glutathione content of *Anabaena variabilis* ARM 441 treated with cartap. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

In control culture, the GSH content increased from 4.5 $\mu\text{g } \mu\text{g}^{-1}$ protein on day 2 to 10.5 $\mu\text{g } \mu\text{g}^{-1}$ protein. In 5, 10 and 20 $\mu\text{g ml}^{-1}$ the glutathione content was 9.5, 8.25 and 6 $\mu\text{g } \mu\text{g}^{-1}$ protein on day 2 which was significantly increased to 26.25, 24 and 17.75 $\mu\text{g } \mu\text{g}^{-1}$ protein on day 8. In *Leptolyngbya foveolarum*, cartap exposure resulted 60, 45 and 30% increase of glutathione content in 20, 40 and 60 $\mu\text{g ml}^{-1}$ respectively after exposure of 192 h (Singh et al., 2014). Anilofos treatment (10 and 20 $\mu\text{g ml}^{-1}$) to *Synechocystis sp. strain* PUPCCC 64 inhibited the GSH content by 19 and 24% (Singh et al., 2013). In rice field Cyanobacterium *Nostoc muscorum*, glutathione synthesis was inhibited in concentration dependent manner upon molinate treatment (0.75 to 2.0 mM) (Galhano et al., 2011a). Lowering of glutathione levels in *Anabaena variabilis* indicates that it might be helping the organism to decrease level of hydrogen peroxide.

4.1.9.2 Ascorbate

Cartap affected the ascorbate level in cyanobacterium in a concentration and time dependent manner. The test microorganism showed 3.75, 6.25, 7.63 μg ascorbate mg^{-1} protein in 5, 10, 20 μg ml^{-1} of cartap, respectively on 8th day (Fig. 4.27). A decrease in ascorbic acid content by 69.07, 48.45, and 37.11% was observed at 5, 10 and 20 μg ml^{-1} of insecticide, respectively with respect to control cultures on the 8th day.

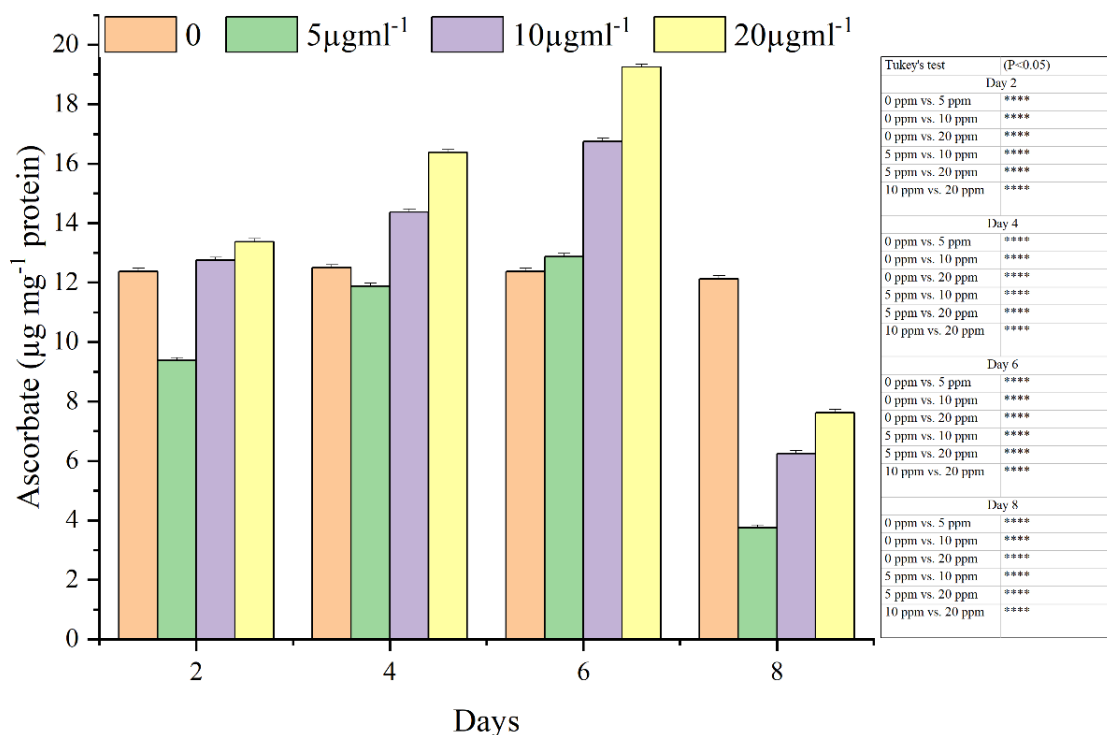


Fig. 4.27: Analysis of ascorbate content of *Anabaena variabilis* ARM 441 treated with cartap. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Similar observations were reported when *Plectonema boryanum* was grown under endosulfan stress showed decreased ascorbate content (Prasad et al., 2005). Anilofos treatment from 1.25 to 5 μg ml^{-1} significantly inhibited ascorbate in dose dependent manner in *Anabaena torulosa* (Singh et al., 2012a). In *L. foveolarum*, 20, 40 and 60 μg ml^{-1} of cartap inhibited the ascorbate content by 48, 56 and 75% respectively (Singh et al., 2014).

4.1.10 Stress indicators

4.1.10.1 Super oxide radicals (SOR) and hydrogen peroxide (H₂O₂)

The amount of SOR and H₂O₂ production was comparatively higher in 20 µg ml⁻¹ cartap than 5 and 10 µg ml⁻¹ of insecticide. In *Anabaena variabilis* there was increase in the production of SOR by 145.02, 152.48 and 167.64% at 5, 10 and 20 µg ml⁻¹ of cartap respectively with respect to control (Fig. 4.28). However, hydrogen peroxide production in control cultures remained constant (13.82 µmol mg⁻¹ protein). The test cyanobacterium produced 17.05, 18.52 and 19.11 µmol H₂O₂ mg⁻¹ of protein at 5, 10 and 20 µg ml⁻¹ of cartap, corresponding to an increase by 23.37, 34 and 38.27% respectively (Fig. 4.29).

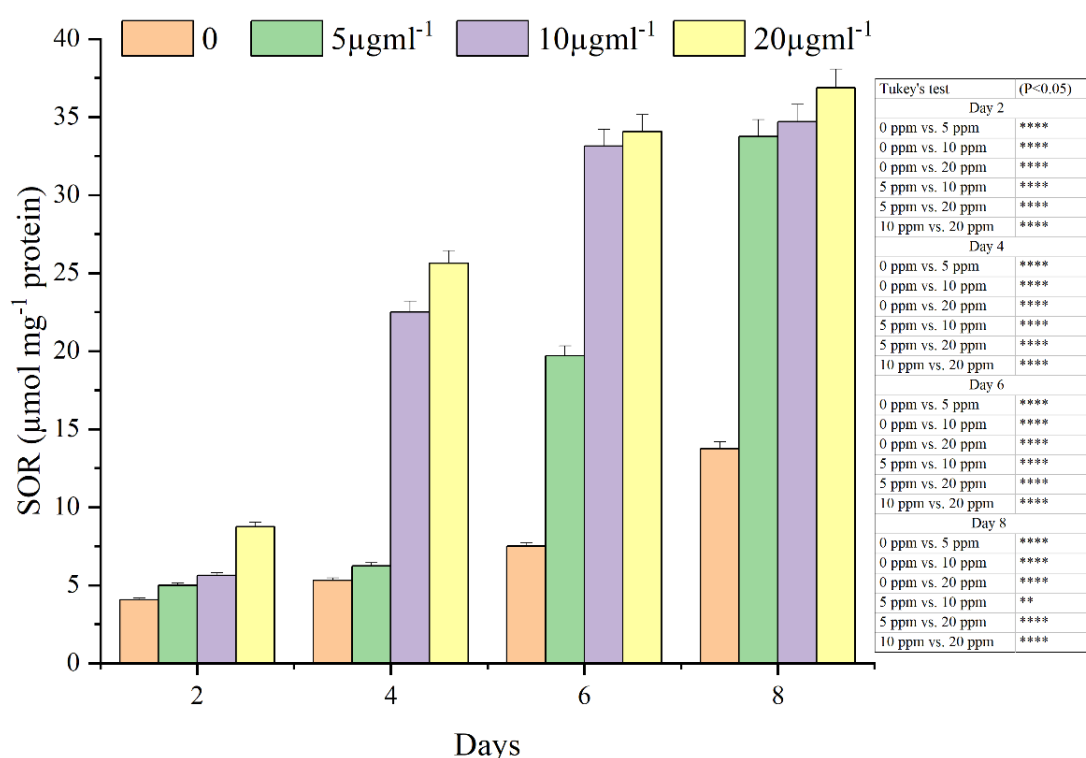


Fig. 4.28: Analysis of cartap effect on super oxide radicals (SOR) of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

In cyanobacteria, environmental contaminants and their metabolic by products can cause oxidative stress and damage. Due to insecticide toxicity, free radicals are formed which results in activation of antioxidant defence system (enzymatic and non enzymatic) of cyanobacteria (Galhano et al., 2010, 2011a, b; Singh et al., 2010, 2012a, 2013). Superoxide radicals (SOR) and hydrogen peroxide (H₂O₂) are important ROS molecules for measuring

the stress caused by insecticide. In all photosynthetic and respiring cells, partial reduction of oxygen molecules continuously produces reactive oxygen species (ROS) (Manikar et al., 2013; Kasting et al., 2002).

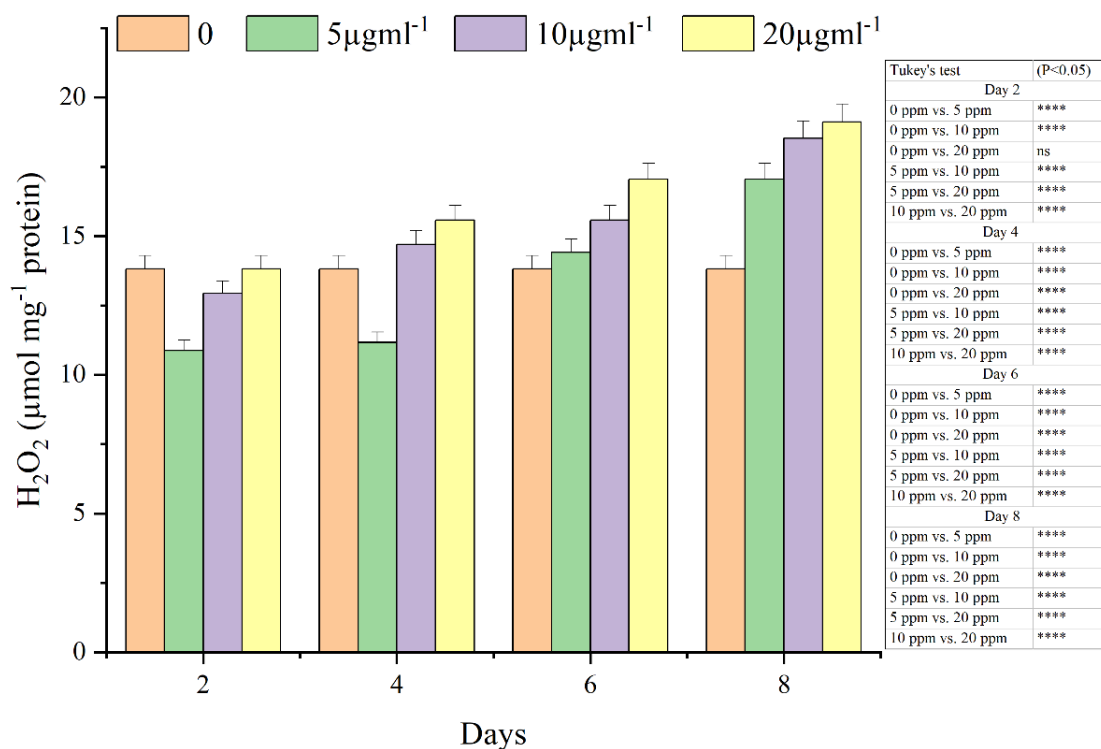


Fig. 4.29: Analysis of cartap effect on hydrogen peroxide (H_2O_2) of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Thylakoid membrane is the major site of SOR production in cyanobacteria and H_2O_2 is formed due to continued reduction of SOR (Lesser, 2006). In our study, the amount of SOR and H_2O_2 production was comparatively higher in 20 $\mu g\ ml^{-1}$ cartap than 5 and 10 $\mu g\ ml^{-1}$ of insecticide (Fig. 4.28, 4.29). UV-B alone and combination with glyphosate, DCMU and 2, 4-D resulted in increased ROS production between 36-136% in *Anabaena* and 22-103% in *Microcystis viridis* (Chen et al., 2012). It has been reported the increase in H_2O_2 production in *Nostoc muscorum* due carbaryl (5-50 $\mu g\ ml^{-1}$) (Bhunja et al., 1994). Paraquat at two different concentrations (0.5 and 0.75 μM) resulted in increased production of ROS in *Chlorella vulgaris* by 1.4 and 2.9 times as compared to control cultures (Qian et al., 2009).

In *Nostoc muscorum* copper stress (2 and 5 μM) resulted in increased ROS production by 131 and 240% while H_2O_2 production was stimulated by 90 and 149% while in *Phormidium foveolarum*, when treated with two different concentrations of copper (2 and 5 μM) resulted in ROS increase by 92 and 242% resulted in enhanced synthesis of H_2O_2 by 78 and 185% (Sheeba et al., 2011). In *Leptolyngbya foveolarum*, cartap stress leads to increased synthesis of ROS by 76, 61 and 53% in 20, 40 and 60 $\mu\text{g ml}^{-1}$ of cartap treated cultures after 192 h. H_2O_2 was also increased by 41, 30 and 22% in 20, 40 and 60 $\mu\text{g ml}^{-1}$ after cartap treatment (Singh et al., 2014).

4.1.10.2 Malondialdehyde (MDA)

Malondialdehyde (MDA) is end product of lipid peroxidation and also used as a stress indicator of cellular membrane system (Manikar et al., 2013). Cartap significantly affected MDA content in ARM 441. MDA content increased from 0.182 $\mu\text{mol mg}^{-1}$ protein on the 2nd day to 0.350 $\mu\text{mol mg}^{-1}$ protein on the 8th day in 5 $\mu\text{g ml}^{-1}$ of insecticide. In 10 $\mu\text{g ml}^{-1}$ cartap the MDA content increased from 0.279 $\mu\text{mol mg}^{-1}$ protein on 2nd day 0.380 $\mu\text{mol mg}^{-1}$ protein on 8th day while an increase of 0.290 to 0.404 $\mu\text{mol mg}^{-1}$ protein on 8th day in 20 $\mu\text{g ml}^{-1}$ treatment was observed. The organism showed 20.68, 31.03 and 39.31% increase in MDA content in cartap treatment compared to control cultures, respectively, on 8th day (Fig. 4.30)

Cartap significantly affected the malondialdehyde (MDA) content, which is signature of oxidative stress in a dose dependent manner in *Anabaena variabilis* by increasing its amount by 20.68, 31.03 and 39.31% at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap. Similar effect was observed when *Anabaena variabilis*, *Aulosira fertilissima* and *Nostoc muscorum* were treated with endosulfan (Kumar et al., 2008a). Prasad et al. (2005) reported an increase in MDA level in *Plectonema boryanum* with endosulfan treatment.

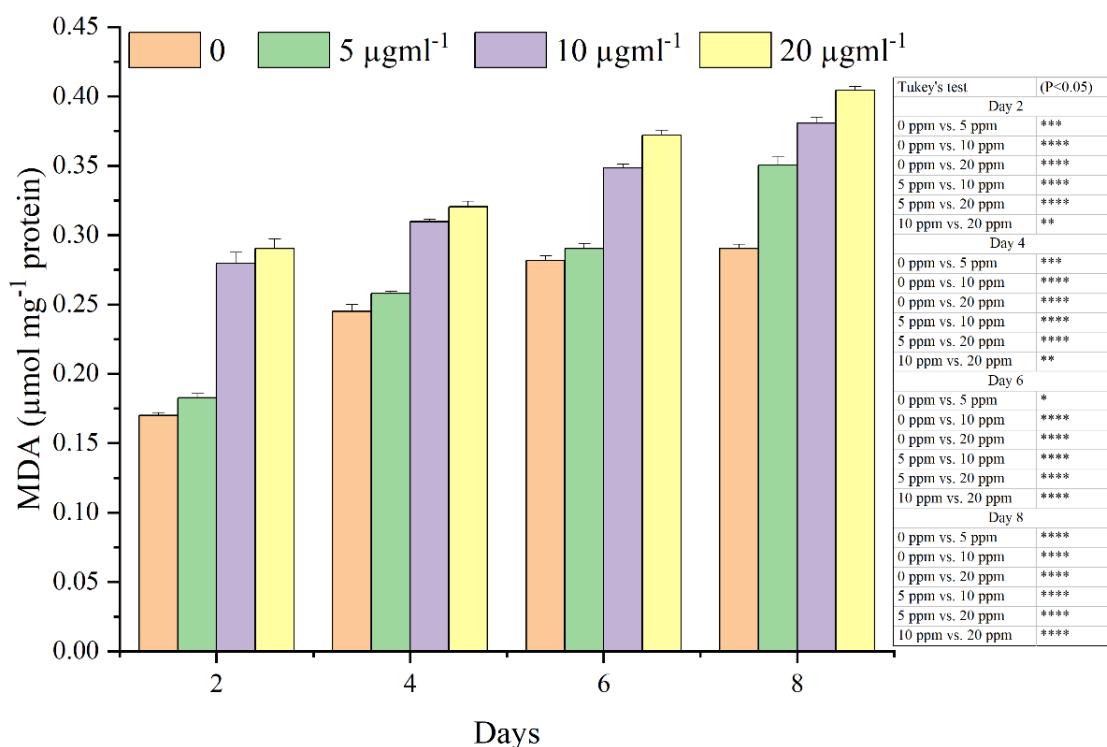


Fig. 4.30 Analysis of cartap effect on malondialdehyde (MDA) content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Treatment with pesticides such as 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU), glyphosate and 2,4-D at 10 μ M along with UV-B significantly increased MDA level in *Anabaena* and *Microcystis viridis* (Chen et al., 2012). MDA content of *Leptolyngbya foveolarum* was increased by 70, 60 and 45% in 20, 40 and 60 μ g ml⁻¹ after 192 h due to cartap treatment (Singh et al., 2014). Cypermethrin (0.001-0.1 μ g ml⁻¹) enhanced the level of MDA in the initial 24 h in *Scrippsiella trochoidea*, *Skeletonema costatum* and *Chattonella marina* and afterwards it was decreased with time (Wang et al., 2012). Malathion treatment (25-100 μ g ml⁻¹) for 20 days increased the level of MDA in *Anabaena variabilis* as reported by Manikar (Manikar et al., 2013). *Nostoc muscorum* when treated with bentazon resulted in increased MDA levels suggesting production of free radicals which help in eliciting toxicity (Galhano et al., 2011). Malathion treatment to *Anabaena sphaerica* increased MDA content between 1.97-2.5 fold (Chakraborty et al., 2017).

4.1.11 Osmolytes

Osmolytes are organic compounds such proline and sucrose, which are accumulated by many plants and animals including micro-organisms to protect themselves from various stresses (Bohnert et al., 1996; Manikar et al., 2013). It was observed that both of these osmolytes are induced in *Anabaena variabilis* ARM 441 by cartap.

4.1.11.1 Proline

Proline is accumulated in cells under stress conditions due to its fast synthesis or decreased rate of degradation (Bohnert et al., 1996). Proline content in *Anabaena variabilis* was increased in dose dependent manner. Proline was significantly increased by 8.66, 25.06 and 32.8% at 5, 10, and 20 $\mu\text{g ml}^{-1}$ cartap with respect to control on day 8 (Fig. 4.31). In 5, 10 and 20 $\mu\text{g ml}^{-1}$ proline content was 1.24, 1.35, 1.41 $\mu\text{g mg}^{-1}$ protein on day 2 which was increased to 1.63, 1.87 and 1.99 $\mu\text{g mg}^{-1}$ protein on day 8.

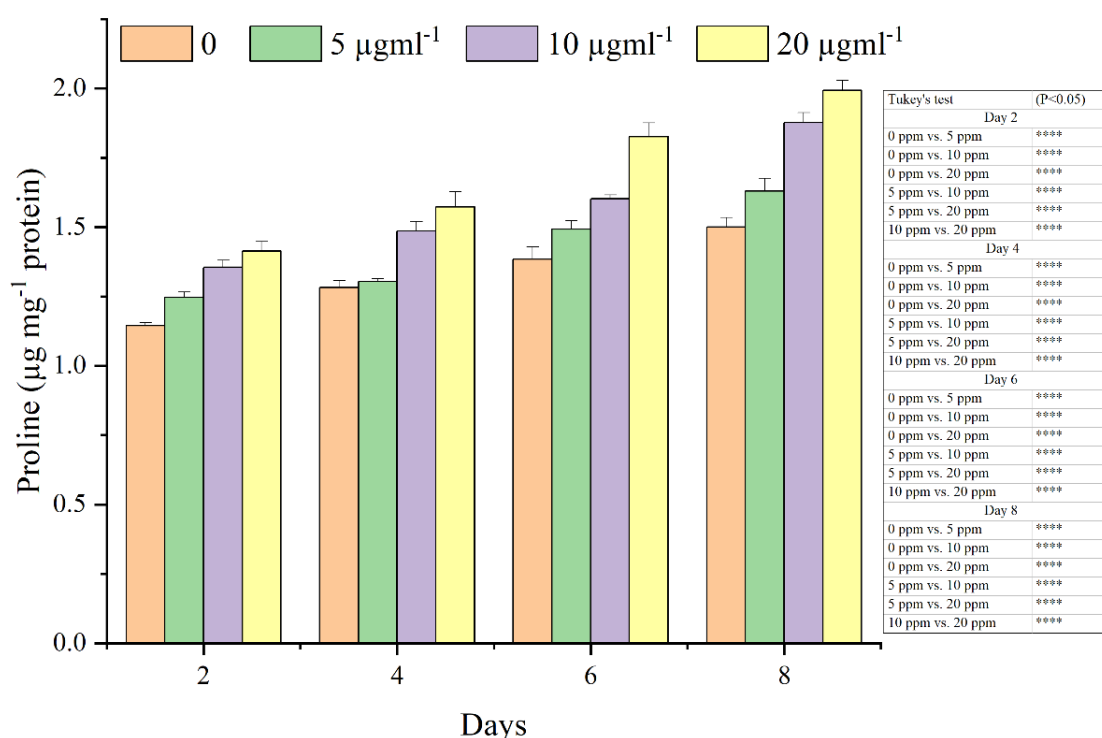


Fig. 4.31: Effect of cartap on proline content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Proline act as an osmolyte, an essential amino acid, important antioxidant as well as helps to stabilize sub-cellular structures (e.g., membranes and proteins), helps in buffering cellular

redox potential and scavenging free radicals under stress conditions (Ashraf and Foolad, 2007). Proline free radical scavenging mechanism has been reported in *Westiellopsis prolifica*-Janet strain-NCCU331, *Spirulina platensis*-S5, *Anabaena variabilis*, *Nostoc muscorum* and *Aulosira fertilissima* in the presence of alphamethrin, endosulfan and deltamethrin; heavy metals and salt stress (Fatma et al., 2007; Choudhary et al., 2007). Involvement of proline as a free radical scavenger has also been reported when *Nostoc muscorum* was treated by molinate (Galhano et al., 2011).

4.1.11.2 Sucrose

Sucrose content in the cyanobacterium was increased in dose and time dependent manner. In 5, 10 and 20 $\mu\text{g ml}^{-1}$ sucrose content was 8.98, 9.62, and 9.45 $\mu\text{g ml}^{-1}$ on day 2 which was increased to 237, 239.5 and 279.5 $\mu\text{g ml}^{-1}$ protein on day 8. Sucrose content was gradually increased by 61.22, 62.92, and 90.13% in 5, 10 and 20 $\mu\text{g ml}^{-1}$ respectively after 8 days. (Fig. 4.32). Accumulation of sucrose as the main organic osmolyte has been reported in *Anabaena* sp. (Manikar et al., 2013).

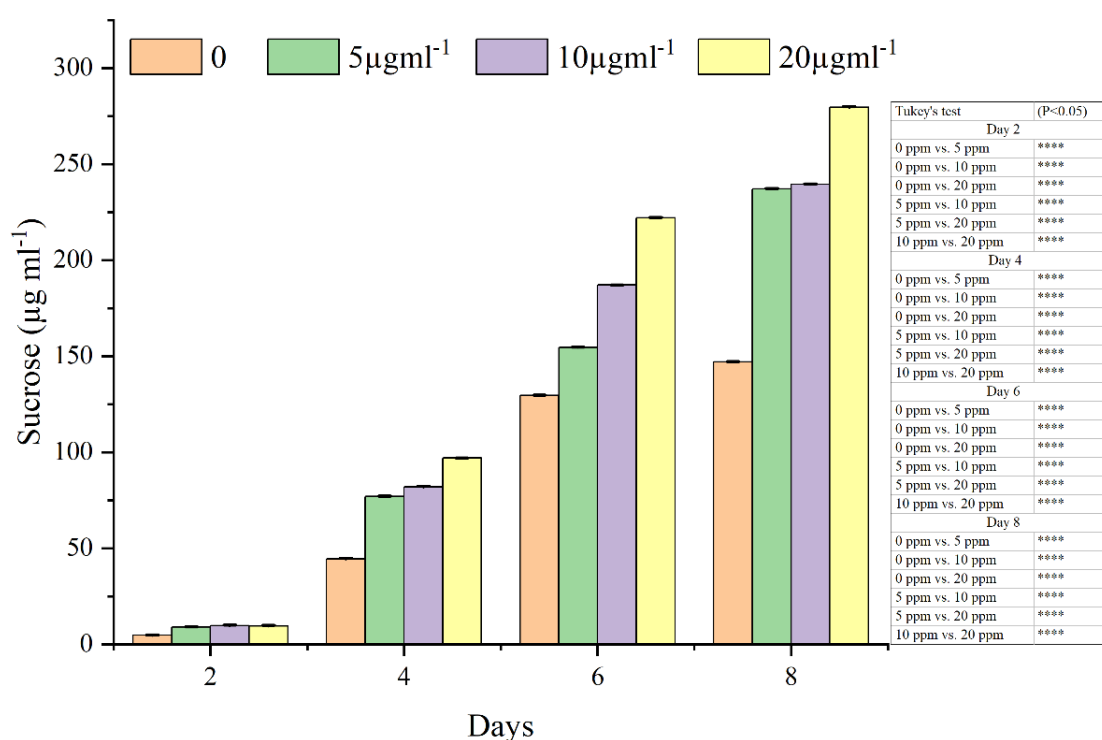


Fig. 4.32: Effect of cartap on sucrose content of *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

Sucrose content was increased when *Anabaena variabilis* was exposed to malathion and accumulated as main organic osmolyte. Different stresses which cause ROS accumulation either directly or indirectly can be associated with accumulation of soluble sugar, which is the most common adaptive stress response in higher plants (Manikar et al., 2013).

4.1.12 Cartap uptake study

To check whether the cyanobacterium was resistant to cartap or utilized it intracellularly, cyanobacterium was grown in medium supplemented with cartap ranging from 5, 10, 30 $\mu\text{g ml}^{-1}$. The samples were collected at specific time intervals upto 24 h showed that cartap was significantly taken up by the cyanobacterium.

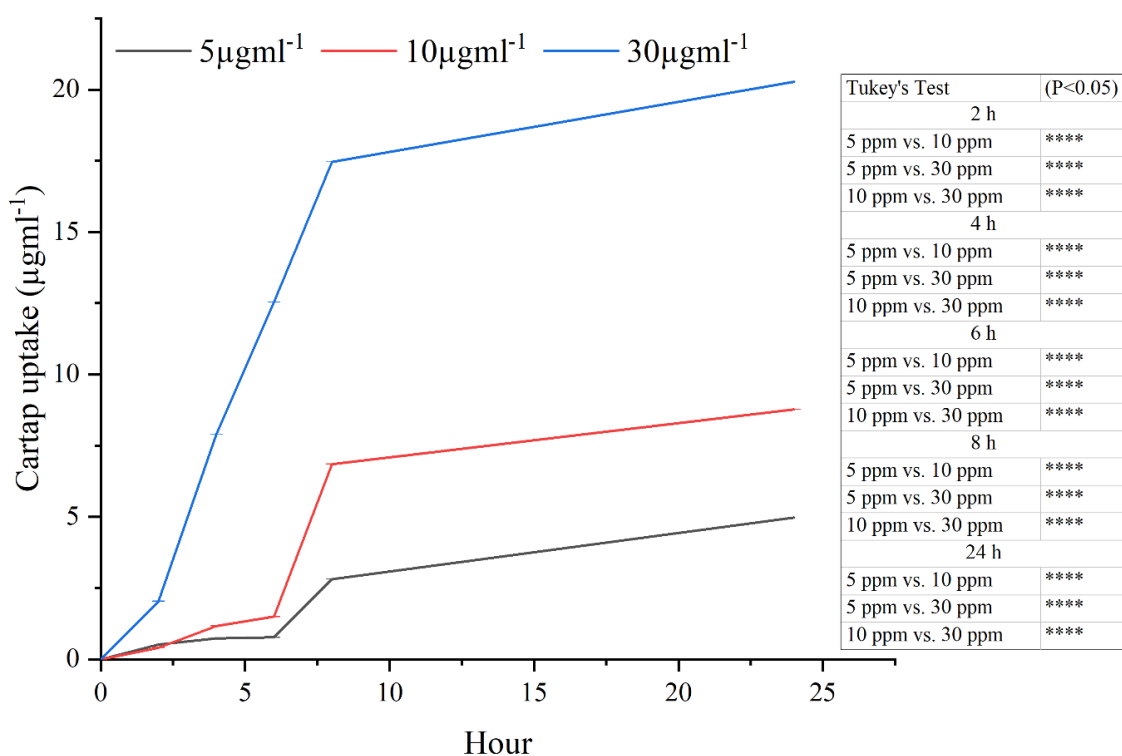


Fig. 4.33: Cartap uptake by *Anabaena variabilis* ARM 441. Values are mean of three independent experiments $\pm$ SE. The asterisk represents significant difference among control and cartap treated cyanobacterium at different time interval based on analysis of variance (two-way ANOVA) followed Tukey's test (*P < 0.05, **P < 0.01, ***P < 0.001).

After 24 h, cultures were collected, centrifuged and washed with distilled water two times. Cartap was estimated in biomass, supernatant and cell wash (Table 4.1). The residual amount of cartap in supernatant was 0.0192 $\mu\text{g ml}^{-1}$ in 5 $\mu\text{g ml}^{-1}$, 1.23 in 10 $\mu\text{g ml}^{-1}$, and 9.73 in 30 $\mu\text{g ml}^{-1}$ after 24 h. In cell wash, very negligible amount of 0, 1, 1.57 $\mu\text{g ml}^{-1}$ cartap was detected in 5, 10, 30 $\mu\text{g ml}^{-1}$. In cell biomass, 1.65, 2.19 and 3.57 $\mu\text{g ml}^{-1}$ was recovered from

5, 10, 30 $\mu\text{g ml}^{-1}$ treated cultures (Fig. 4.33). The total amount of cartap recovered from all these components was 1.65, 4.42, 14.87 $\mu\text{g ml}^{-1}$ in 5, 10, 30 $\mu\text{g ml}^{-1}$ representing percentage removal of 67, 55.8, and 50.43% after 24 h. This means that rest of cartap in all treated cultures was metabolized by cyanobacterium.

Glyphosate (5-2mM) when supplemented in medium, was intracellularly taken up by two cyanobacterial species *Synechocystis* 6803 and *Anabaena variabilis* (Powel et al., 1991). *Nostoc UAM 2005*, *Gloeotheca PCC 6501*, *Anabaena PCC 7119*, *Chlorogloeopsis PCC 6912* and *Plectonema calothricoides* removed trichlorofon (300 $\mu\text{g ml}^{-1}$) from the medium (Orus and Marco 1991). *Chlorella vulgaris* and *Synechococcus elongates* rapidly removed terbutryn and atrazine from the medium (Gonzalez-Barreiro et al., 2006). *Synechocystis* sp. strain PUPCCC 64 when grown in 5 $\mu\text{g ml}^{-1}$ of chlorpyrifos efficiently metabolized 4.69 $\mu\text{g ml}^{-1}$ within 8 days (Singh et al., 2011). In another report by Singh et al. (2013), *Synechocystis* sp. also uptake anilofos intracellularly and metabolize it upto 120 h. *Nostoc muscorum* metabolized butachlor into 1, 2- benzene dicarboxylic acid and phenol which was confirmed by GC-MS analysis (Anees et al., 2014). *Anabaena fertilissima* used monocrotophos, quinalphos and malathion as phosphorus source after metabolizing it (Subramanian et al., 1994). *Spirulina* sp. mineralized glyphosate and used it as phosphorus source ((Lipok et al., 2007). *Leptolyngbya foveolarum* accumulated cartap intracellularly and degraded it showing that cyanobacterium is using it as nitrogen source (Singh et al., 2014).

Table 4.1: Cartap hydrochloride in supernatant and cell biomass of *Anabaena variabilis* after 24 h of treatment

Components / Cartap	5 $\mu\text{g ml}^{-1}$	10 $\mu\text{g ml}^{-1}$	30 $\mu\text{g ml}^{-1}$
Biomass	1.65 $\pm$ 0.002	2.19 $\pm$ 0.007	3.57 $\pm$ 0.008
Supernatant	0.0192 $\pm$ 0.005	1.23 $\pm$ 0.003	9.37 $\pm$ 0.006
Cell wash	0	1 $\pm$ 0.002	1.57 $\pm$ 0.004
Total	1.65 $\pm$ 0.008	1.42 $\pm$ 0.011	14.87 $\pm$ 0.016

4.1.13 Scanning electron micrography

The FESEM of treated samples was done after the 3rd, 6th and 9th day of treatment with cartap hydrochloride. FESEM analysis revealed that external morphology of *Anabaena variabilis* was disrupted and damaged, when treated with 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap. Untreated cells

presented the distinctive straight, elongated shape and were intact (Fig. 4.34, a-c), whereas cells treated with 5-20 $\mu\text{g ml}^{-1}$ cartap showed morphological changes. The surface of treated cells was deteriorated (Fig. 4.34, d-l). The cells got burst and released cytoplasmic material, which resulted in stacking of one filament over another (Fig. 4.34, e, h). At higher concentration (20 $\mu\text{g ml}^{-1}$) of cartap, the shape of the cells was completely changed and resulted in the formation of akinetes which are thick-walled resting bodies formed under unfavorable environmental conditions (Fig. 4.34, i, l). Filaments collapsed and completely separated from each other indicating the potential adverse effect of cartap on the structural and functional integrity of *Anabaena variabilis*.

SEM analysis revealed that cartap treated filaments of *Anabaena variabilis* showed deteriorated cell surface as compared to untreated ones, which are distinctive straight with elongated shape. At high concentration (20 $\mu\text{g ml}^{-1}$) of cartap, cells of *Anabaena variabilis* collapsed, akinetes are formed and separates from each other indicating the potential adverse effect of cartap on the integrity of cell structure, morphology and metabolic activities. In *Microcystis aeruginosa*, the external morphology remained unaffected and both treated and untreated cells have similar size and characteristic spherical shape when treated with 71.20 mgL^{-1} (IC50) of 2, 4-D (Martinez and Martinez, 2018). Scanning electron micrographs of *Nostoc muscorum* showed swelling of cells followed by complete separation from each other when treated with 80 $\mu\text{g ml}^{-1}$ of butachlor (Martinez et al., 2018).

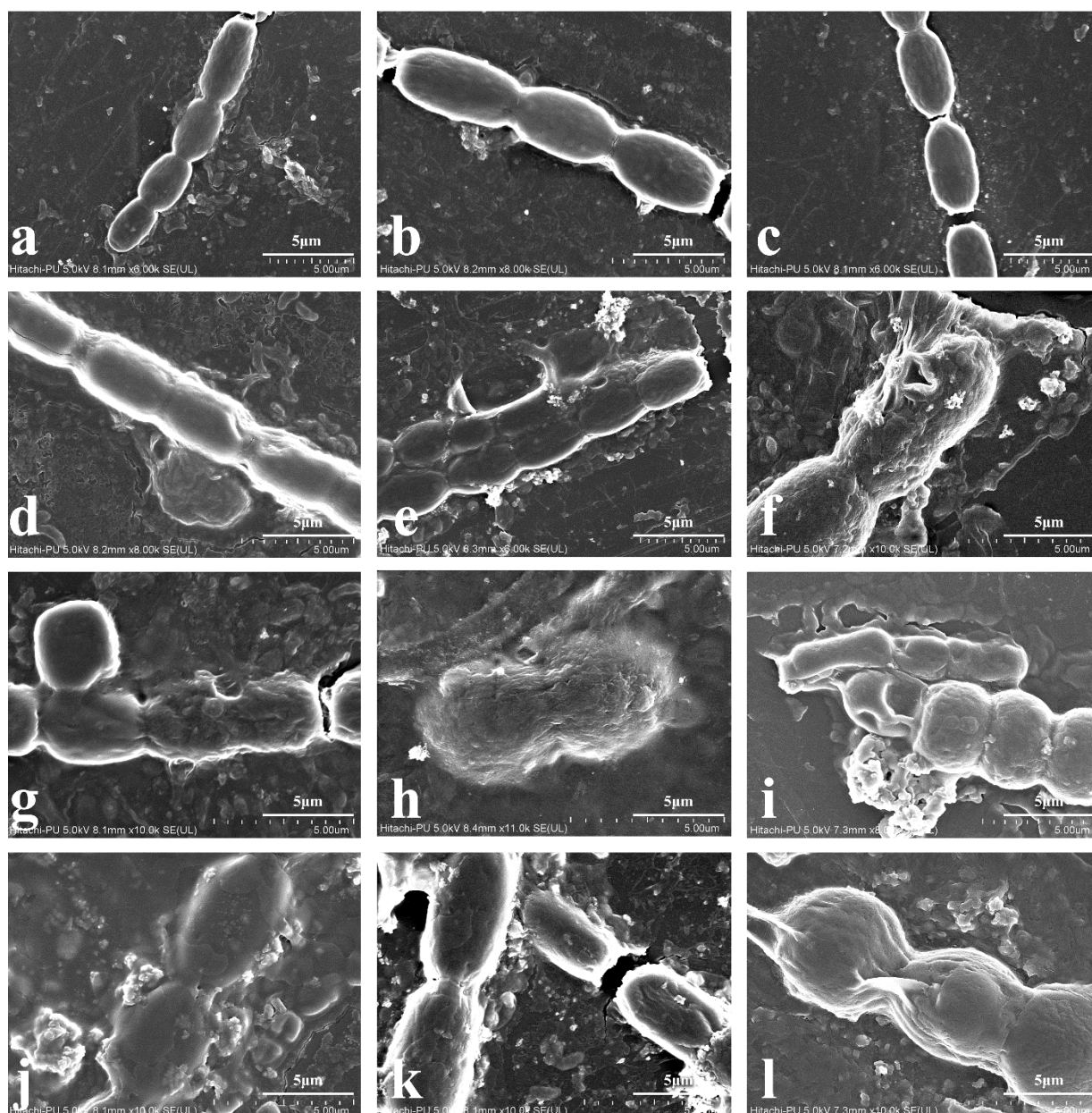


Fig. 4.34: Scanning electron microscopy of *Anabaena variabilis* ARM 441 in presence of 0, 5, 10, 20 µg ml⁻¹ of cartap hydrochloride at different time interval. Control (0 µg ml⁻¹): a, b, c on 3rd, 6th and 9th day; 5 µg ml⁻¹: d, e, f on 3rd, 6th and 9th day; 10 µg ml⁻¹: g, h, i on 3rd, 6th and 9th day; 20 µg ml⁻¹: j, k, l on 3rd, 6th and 9th day

4.3 Nanoformulation of cartap hydrochloride and its characterization

4.3.1 Chitosan tripolyphosphate nanospheres (CS-TPP nps)

4.3.1.1 Synthesis of nanospheres

Chitosan nanospheres were synthesized through ionic gelation method (Gan and Wang, 2007; Gan et al., 2005). In acidic media, chitosan shows polycationic nature and represents -NH^+_{3} sites. Sodium tripolyphosphate (TPP) at pH 4.5 dissociates to form phosphoric and hydroxyl ions. Cationic sites in chitosan are responsible for the cross-linking of chitosan.

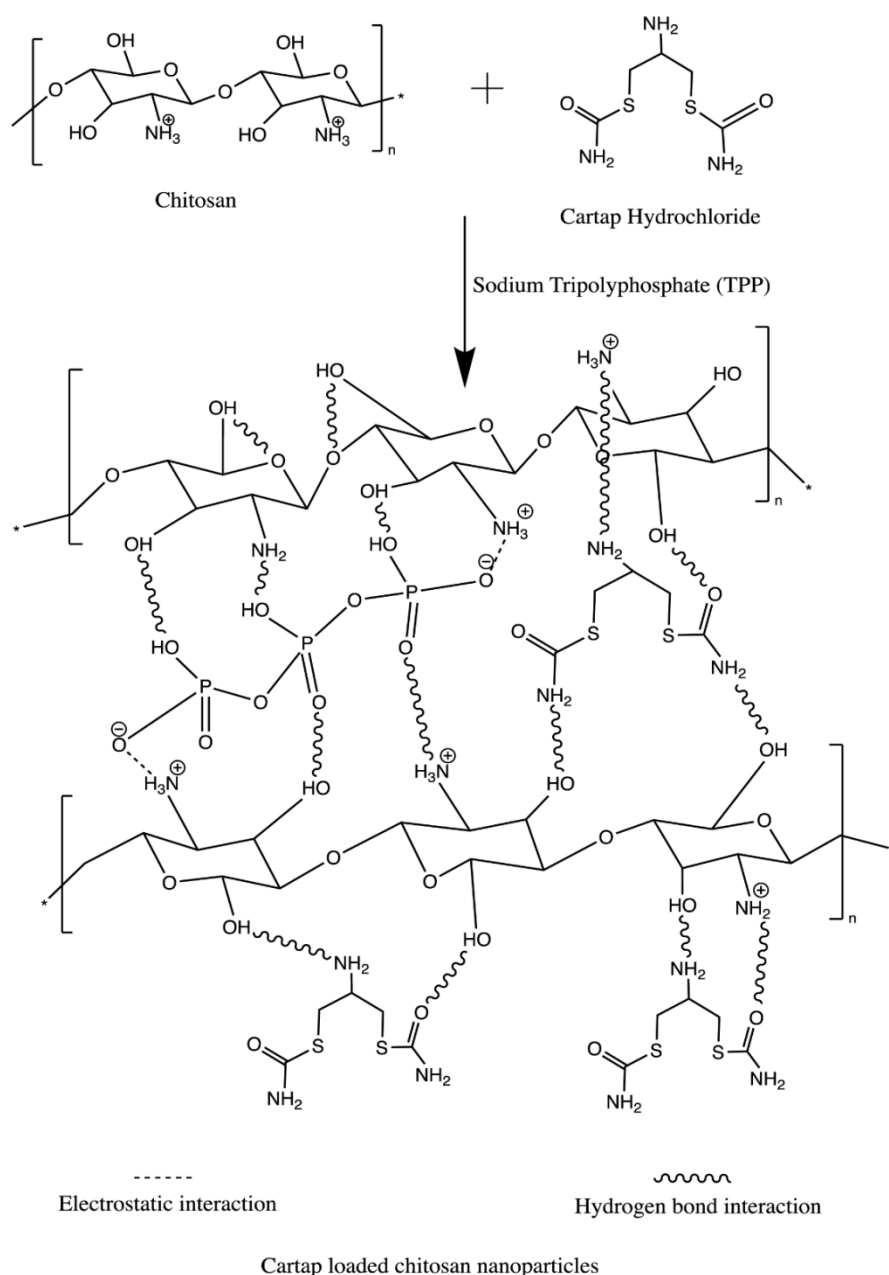


Fig. 4.35: Proposed structure of cartap hydrochloride loaded chitosan tripolyphosphate (C-CS-TPP) nanospheres

Cartap was mixed with chitosan solution under continuous stirring, the hetero atoms of cartap initiates formation of hydrogen bond with protonated chitosan chain to attain higher encapsulation. With the addition of cross-linking agent (TPP) to the chitosan-cartap insecticide cocktail, negatively charged TPP interacts with cartap-chitosan, leading to the synthesis of insecticide loaded chitosan nanospheres.

The phosphoric and OH ions of TPP compete with each other to interact with the NH_3^+ sites of chitosan. Inter- and intramolecular bonds were formed between TPP and amino group of chitosan when these two phases were mixed. The mechanism of interaction in the formulation is given in Fig.4.35. The amino groups of chitosan are responsible for interaction with polyanions by means of inter and intramolecular cross linkages to form nanospheres (Xu and Du, 2003; Vila et al., 2004). It is only the free amino groups on which synthesis of nanospheres depends, as it increases the electrostatic interactions between the insecticide and the nanospheres to reduce the particle size (Yang and Hon, 2009; Janes et al., 2001).

4.3.1.2 Physicochemical characterization and stability of the nanoformulation.

Dynamic light scattering was used to determine the size distributions of the particles containing the insecticide. The average size distributions of the CS-TPP nps using the DLS was 201.35 ± 3.65 nm but after encapsulation of cartap in chitosan nanospheres, the size of C-CS-TPP nps was increased to 263.87 ± 4.58 nm respectively (Fig. 4.36). Similarly, it was observed in case of polydispersity index of nanospheres, the polydispersity values for CS-TPP nps was 0.286 ± 0.002 and in insecticide loaded C-CS-TPP nps it was 0.271 ± 0.004 indicating that size distribution was more homogeneous (lower polydispersity) in cartap C-CS-TPP nps compared to the blank nps. The zeta potential of CS-TPP nps was $+40.96 \pm 1.25$. However, the encapsulation of cartap in the nanospheres resulted in increased stability, with higher zeta potential $+43.6 \pm 0.260$ mV in C-CS-TPP nps.

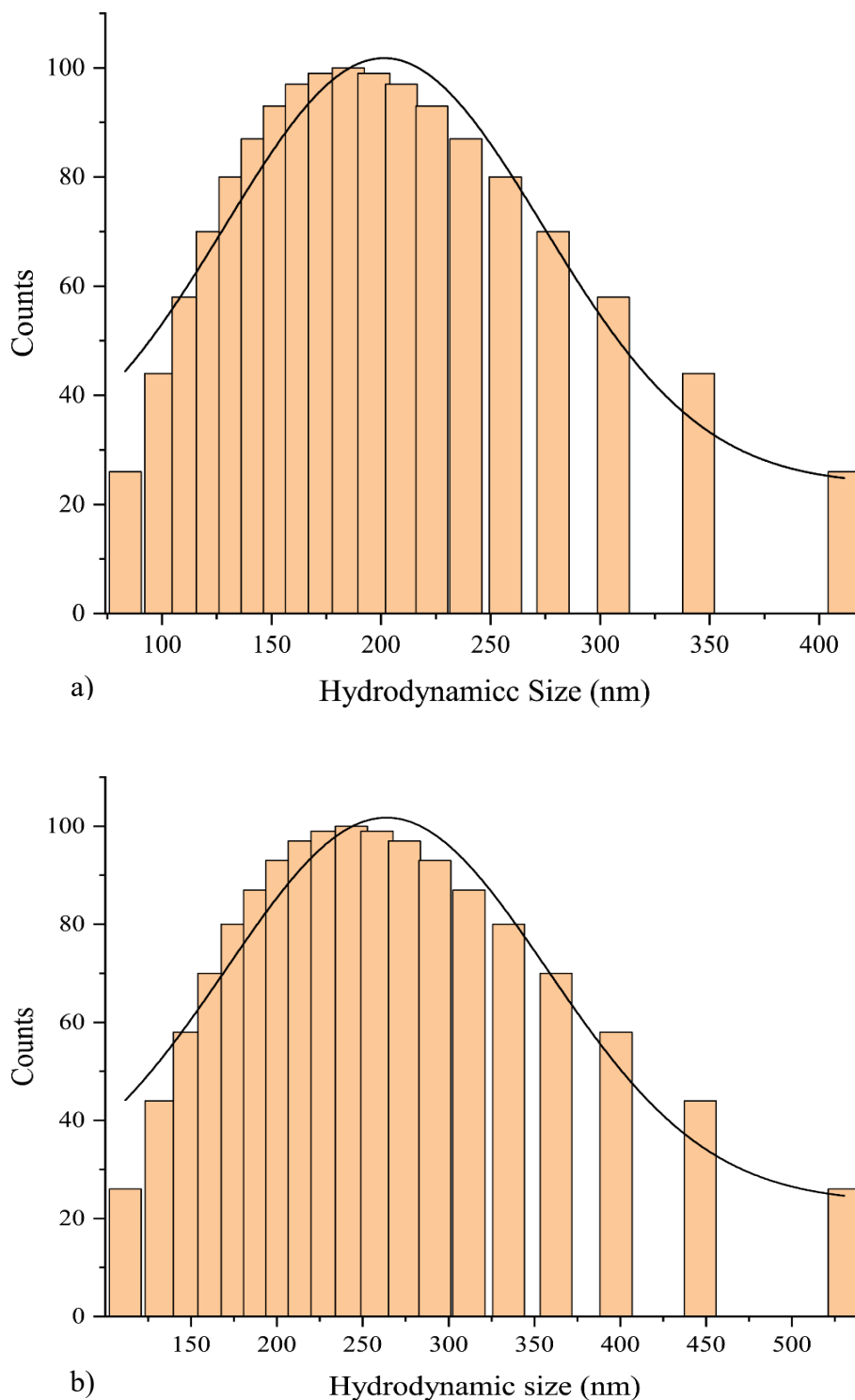
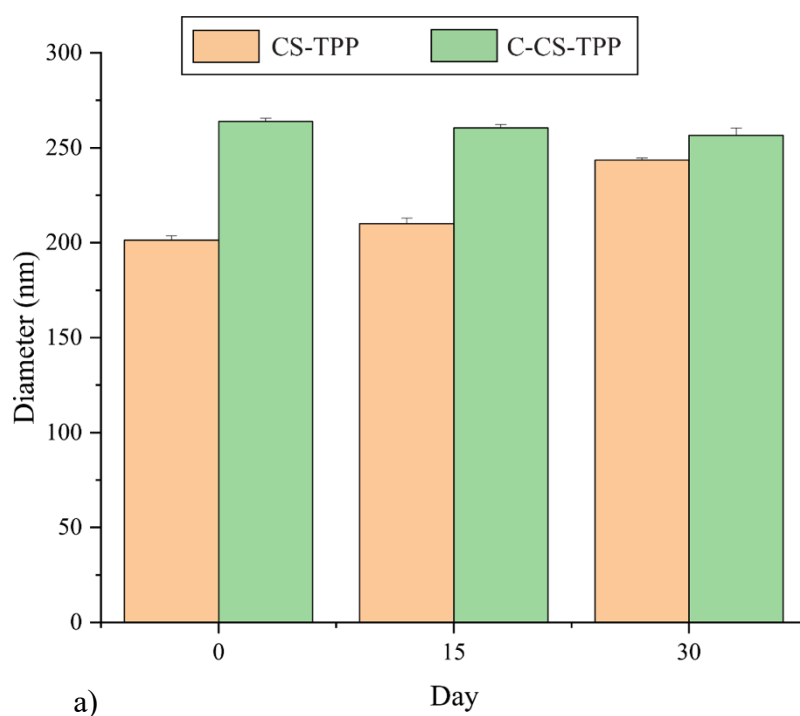


Fig. 4.36: Average size distribution analysis by dynamic light scattering (DLS) of a) chitosan tripolyphosphate nanospheres (CS-TPP); b) cartap loaded chitosan nanospheres (C-CS-TPP)

In a study by Cintia et al. (2016), the average particle size of chitosan tripolyphosphate nanoparticles loaded with imazapyr and imazapic was 478.6 nm and zeta potential of + 26 mV. Insecticide nicotine hydrochloride (NCT) nanoparticles was synthesized by using CS-TPP having size around 300nm with a PDI value of 0.3 and zeta potential value of 45mV

(Yang et al., 2018). The hydrodynamic diameter of paraquat loaded chitosan nanoparticles was around 300 nm. The polydispersion index values was 0.20, indicating that the nanoparticles were monodisperse and homogeneous in terms of size. The values of the zeta potential were positive due to the presence of the cationic chitosan polymer, and were around 40 mV (Grillo et al., 2014a). The hexaconazole incorporated chitosan tpp nanoparticles showed particle size of 100nm with polydispersity value of 0.325 at a chitosan concentration of 0.10% and a TPP concentration of 0.08%. Zeta potential value was found to be +35.3 (Chauhan et al., 2017).

The stability of the blank nanospheres and cartap loaded nanospheres were checked by measuring average size, zeta potential and polydispersity over a period of 30 days (Fig. 4.37). The particles were stable in the presence of insecticide with no change in above parameters. The zeta potential also remained at around 40 mV, which concludes that electrostatic repulsion was maintained between the particles thus minimizing the possibility of aggregation. In case of blank chitosan nanospheres average size, zeta potential and polydispersity values changes indicating particle aggregation. However, the cartap hydrochloride encapsulation in the nanospheres made the particles more stable by increasing their zeta potential. This could be attributed to the interactions between the polymeric chains of the chitosan nanospheres and cartap hydrochloride. Polydispersity values of blank nanospheres were higher than that of insecticide loaded nanospheres.



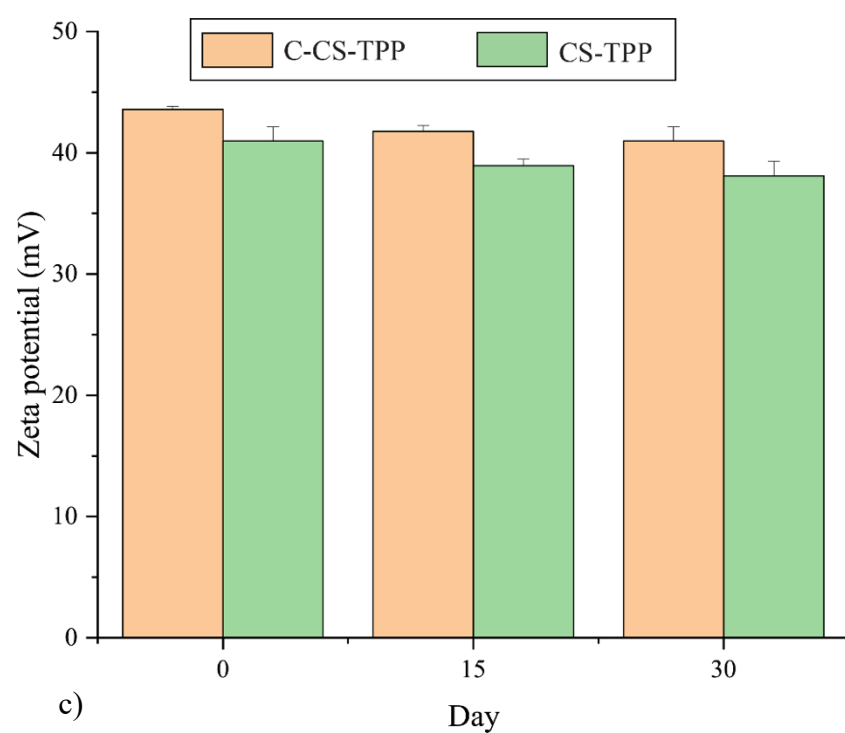
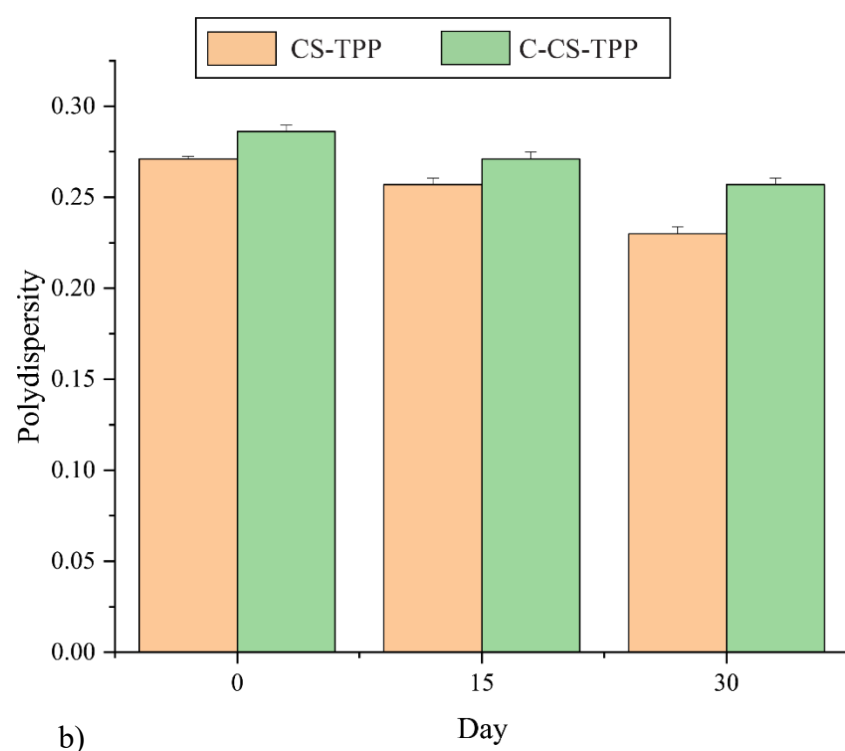


Fig. 4.37: Stability of nanospheres over a period of 30 days. CS-TPP: chitosan tripolyphosphate nanospheres; C-CS-TPP: cartap chitosan tripolyphosphate nanospheres. a) Average size b) Polydispersity c) Zeta potential

Nicotine hydrochloride (NCT) nanoparticles were stable for 45 days at 25°C (Yang et al., 2018). The paraquat loaded chitosan nanoparticles system was stable for 60 days at room temperature (Grillo et al., 2014a). Encapsulation of imazapyr and imazapic in the nanoparticles resulted in increased stability in terms of average size, zeta potential and polydispersity than blank nanoparticles over period of 30 days (Cintia et al., 2016).

4.3.1.2.1 FTIR

Cartap-chitosan interactions of chitosan nanospheres crosslinked with TPP by ionic gelation process were analyzed through FTIR spectroscopy (Fig. 4.38, 4.39). The FTIR spectrum of the chitosan showed bands at 3396.5, 2908, 2879.4, 2166.8, 1663.4, 1381, 1074.2 and 1028.4 cm^{-1} . The broad peak around 3600–3000 cm^{-1} in pure chitosan should be assigned to the stretching vibration of –OH, the extension vibration of the >NH, and the intermolecular hydrogen bonds of the polysaccharide. Characteristic band of the OH stretching and NH stretching vibration was indicated by intense broad peak at 3396.5 cm^{-1} . CH stretching modes (antisymmetric and symmetric) of vibration corresponds to bands at 2908 and 2874 cm^{-1} . Presence of amide groups was confirmed by bands at 1663 cm^{-1} , 1594.6 cm^{-1} , 1320.6 cm^{-1} which corresponds to amide I, II and III (Pawlak and Mucha, 2003; Dong et al., 2002), which indicates that chitosan had a high deacetylation degree. Saccharide peaks of chitosan appeared in the range of 960 cm^{-1} to 1180 cm^{-1} . Asymmetric stretch of C-O-C showed band near 1074.2 cm^{-1} .

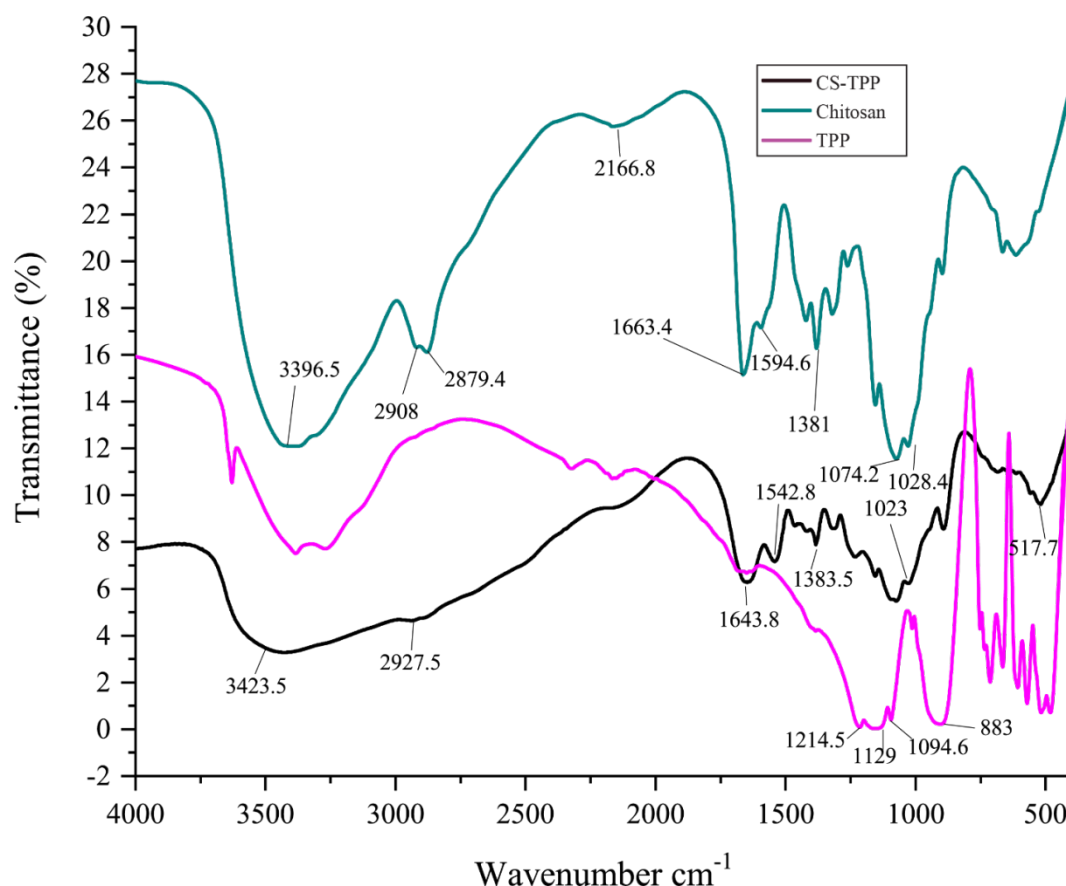


Fig. 4.38: FTIR spectrum of a) Chitosan, sodium tripolyphosphate (TPP) and chitosan nanospheres (CS-TTP)

Blank chitosan nanospheres showed absorption bands at 3423.5, 2927.5, 1643.8, 1542.8, 1383.5, 1023 and 517 cm^{-1} respectively (Fig. 4.38). The OH stretching vibration was shown by broad absorption band at 3423 cm^{-1} . The bands in the spectral region between 3200 cm^{-1} and 3550 cm^{-1} confirmed OH bonding in nanospheres and indicated their hydrophilic nature. Asymmetric stretching vibration of methylene was observed at 2927.5 cm^{-1} in chitosan nanospheres (Jain et al., 2016). NH bending vibration was shown by the absorption band at 1643.8 cm^{-1} (Hashad et al., 2016). Stretching of aliphatic nitro compounds was shown by N–O stretching which was assigned by bands at 1542.8 cm^{-1} and 1383.5 cm^{-1} (Krajewska, 2005). The CO stretching band of chitosan at 1663.4 cm^{-1} was shifted to 1643.8 cm^{-1} in case of nanospheres, indicating the interaction between chitosan nanospheres (Jayakumar et al., 2011). The band for CO stretching vibration of primary alcohol was observed at 1023 cm^{-1} . Bromoalkanes show absorption band at 517 cm^{-1} .

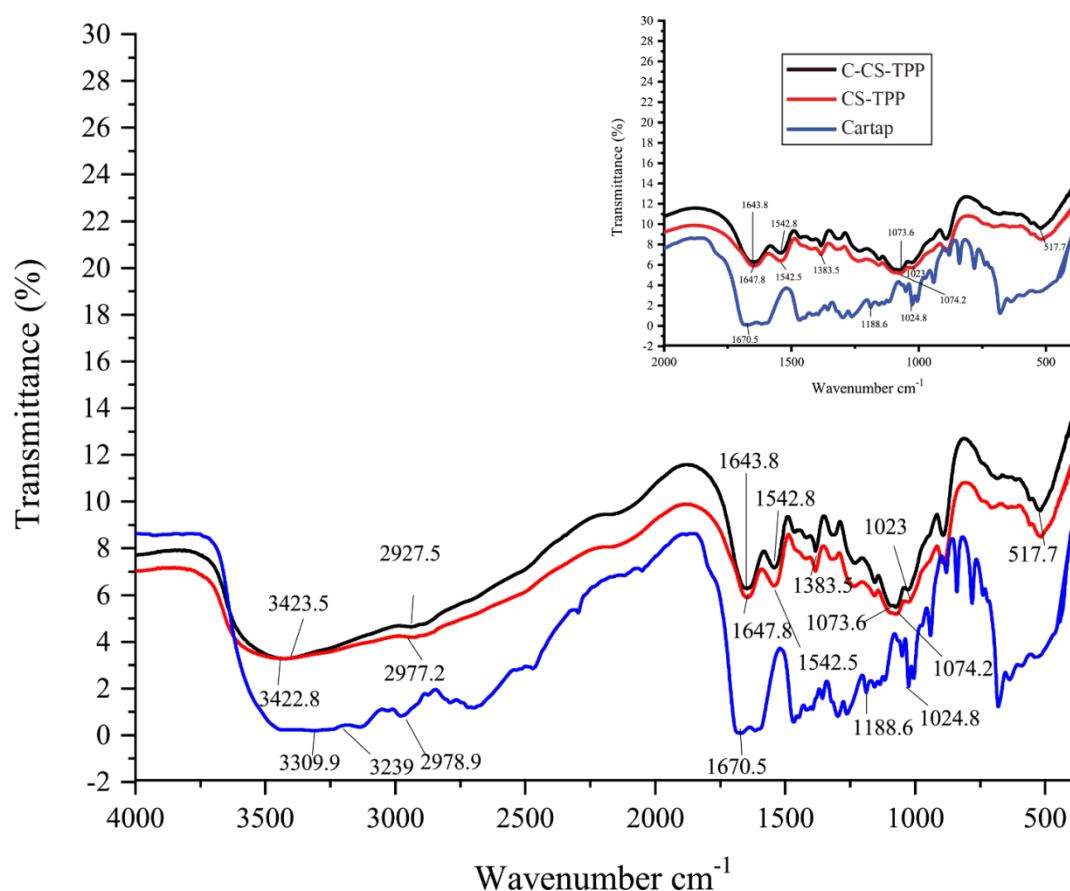


Fig. 4.39: FTIR spectrum of Cartap, chitosan nanospheres (CS-TPP) and cartap loaded chitosan nanospheres (C-CS-TPP)

The FTIR spectrum of CS-TPP nps and C-CS-TPP nps (Fig. 4.39) were consistent with previously published data (Chauhan et al., 2017). The comparative spectra, in case of CS – TPP nanospheres, are presented in the areas of hydroxyl and carbonyl groups absorptions, respectively, where several shifts of the characteristic peaks were observed. O H and N H stretching modes of interaction was shown by broad band at 3423.5 cm^{-1} in case of blank nanospheres but this bond get shifted to 3422.8 cm^{-1} in insecticide loaded nanospheres suggesting the existence of both hydrogen bond and electrostatic interactions (Xu and Du, 2003; Qin et al., 2006; Dudhani and Kosaraju, 2010; Wu et al., 2005; Gomathi et al., 2017; Lazaridou et al., 2020). These shifts are clearly due to the ionic interaction taking place between CS and TPP for the formation of nanospheres. Non-bonding interactions of CO groups were also observed during nanospheres synthesis as band was shifted to 1643.8 and 1647.8 cm^{-1} from 1663.4 cm^{-1} for blank CS-TPP nps and cartap C-CS-TPP nps (Rejinold et al., 2011). Cartap also has two peaks in these areas as at 1670.5 and 1615.8 cm^{-1} . It is characteristic that in all C-CS-TPP nanospheres these peaks were not recorded and it seemed that the high intensity peak at 1670.5 cm^{-1} was shifted to lower wavenumbers and merged

with the carbonyl absorption of CS-TPP forming a new peak with absorption at 1647.8 cm^{-1} . This finding indicates that the carbonyl peaks of cartap also participated in hydrogen bond interactions with CS (Lazaridou et al., 2020).

The band for NH_2 bending vibration was shifted to 1542.5 and 1542.8 cm^{-1} from 1594.6 cm^{-1} for blank CS-TPP nps and C-CS-TPP nps respectively which was in accordance with previous work which proved that this shift occurs due to interaction of amino group and phosphate anion (Xu and Du, 2003). $\text{P}=\text{O}$ stretching and $\text{P}-\text{O}$ bending vibration were also shown by nanospheres. Peaks at 1234.1 and 1231.9 cm^{-1} shows $\text{P}=\text{O}$ stretching and peaks at 1073.6 cm^{-1} and 1074.2 cm^{-1} was indicative of $\text{P}-\text{O}$ bending vibration in blank CS-TPP nps and C-CS-TPP nps (Mathew et al., 2010). It can be concluded that peak shifting of different functional groups such as CO, NH and OH and the appearance of a new peak for $\text{P}=\text{O}$ and $\text{P}-\text{O}$ as compared to spectrum of pure chitosan, confirmed that chitosan nanospheres were formed (Knaul et al., 1999; Wang et al., 2001).

Cartap alone showed major peaks (Fig. 5b) at 1670.5 cm^{-1} (C O stretching), 3309.9 cm^{-1} and 3239 cm^{-1} (NH stretching), 2978.9 cm^{-1} (NCH_3 stretching) and 1188.6 cm^{-1} to 1024.8 cm^{-1} (CS stretching). Furthermore, it was noted that all these peaks of cartap were absent in synthesized cartap nanospheres, while the peak at 1383 cm^{-1} was still present. The absence of the characteristic peaks of cartap indicated the interaction of cartap with chitosan. Almost all the absorption peaks of blank chitosan nanospheres and cartap chitosan nanospheres overlapped with each other explaining no strong covalent interaction existed between cartap and chitosan.

4.3.1.2.2 FESEM

FESEM micrographs of the blank and cartap loaded chitosan nanospheres (Fig. 4.40) revealed that the synthesized formulations were in the nano size range and spherical in shape. The size of blank chitosan nanospheres were in range of 117.01 - 185.27 nm (Fig. 4.40a) and cartap loaded samples were found in the range of 163.50 - 276.74 nm (Fig. 4.40b). The image also revealed that the size of cartap loaded nanoparticle is increased after encapsulation and these measurements were supported by DLS studies.

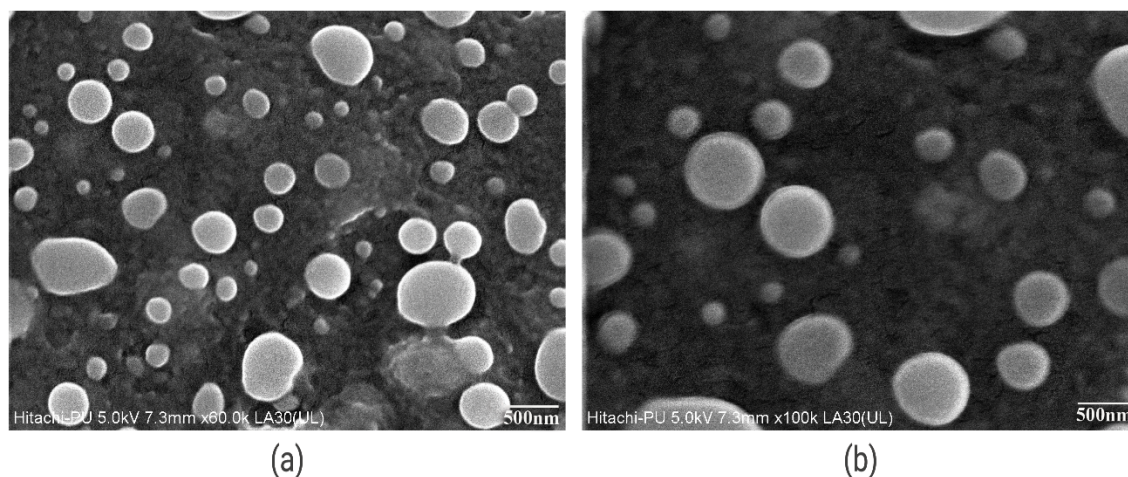


Fig. 4.40: FESEM micrographs of a) chitosan nanospheres (CS-TPP); b) cartap loaded chitosan nanospheres (C-CS-TPP)

4.3.1.2.3 XRD

Diffraction pattern of cartap, chitosan, TPP, blank chitosan nanospheres and cartap loaded chitosan nanospheres synthesized with TPP crosslinker is shown in Fig. 4.41. X-ray diffractogram of TPP powder, showed distinctive sharp peaks at a diffraction angle of $2\theta \approx 11.13^\circ, 18.5^\circ, 21.8^\circ, 22.9^\circ, 27.5^\circ, 27.9^\circ, 31.8^\circ, 32.5^\circ, 36.62^\circ, 37.1^\circ, 38.3^\circ$ which confirmed the crystalline nature of crosslinking agent. Chitosan powder also showed crystalline nature as it showed diffraction at around $2\theta \approx 20^\circ$. Sharp peaks were observed at $2\theta \approx 11.3^\circ, 13.7^\circ, 14.5^\circ, 16.4^\circ, 19.0^\circ, 22.5^\circ, 24.3^\circ, 26.5^\circ, 28.8^\circ, 30.5^\circ, 31.9^\circ, 34.3^\circ, 35.9^\circ, 37.9^\circ, 38.9^\circ, 45.9^\circ, 46.8^\circ, 48.2^\circ$ in case of pure cartap, indicating its crystalline nature. The insecticide loaded chitosan nanospheres C-CS-TPP nps showed diffraction peaks at $2\theta \approx 31.9^\circ, 45.5^\circ, 23.74^\circ, 11.37^\circ$ and 18.0° . The broadening of diffraction peaks at $2\theta \approx 31.9^\circ, 45.5^\circ$ confirmed the amorphous nature of insecticide nanospheres (Fajardo et al., 2010). The change arrangement of molecules in the crystal lattice resulted in different diffraction patterns of chitosan and cross-linked chitosan.

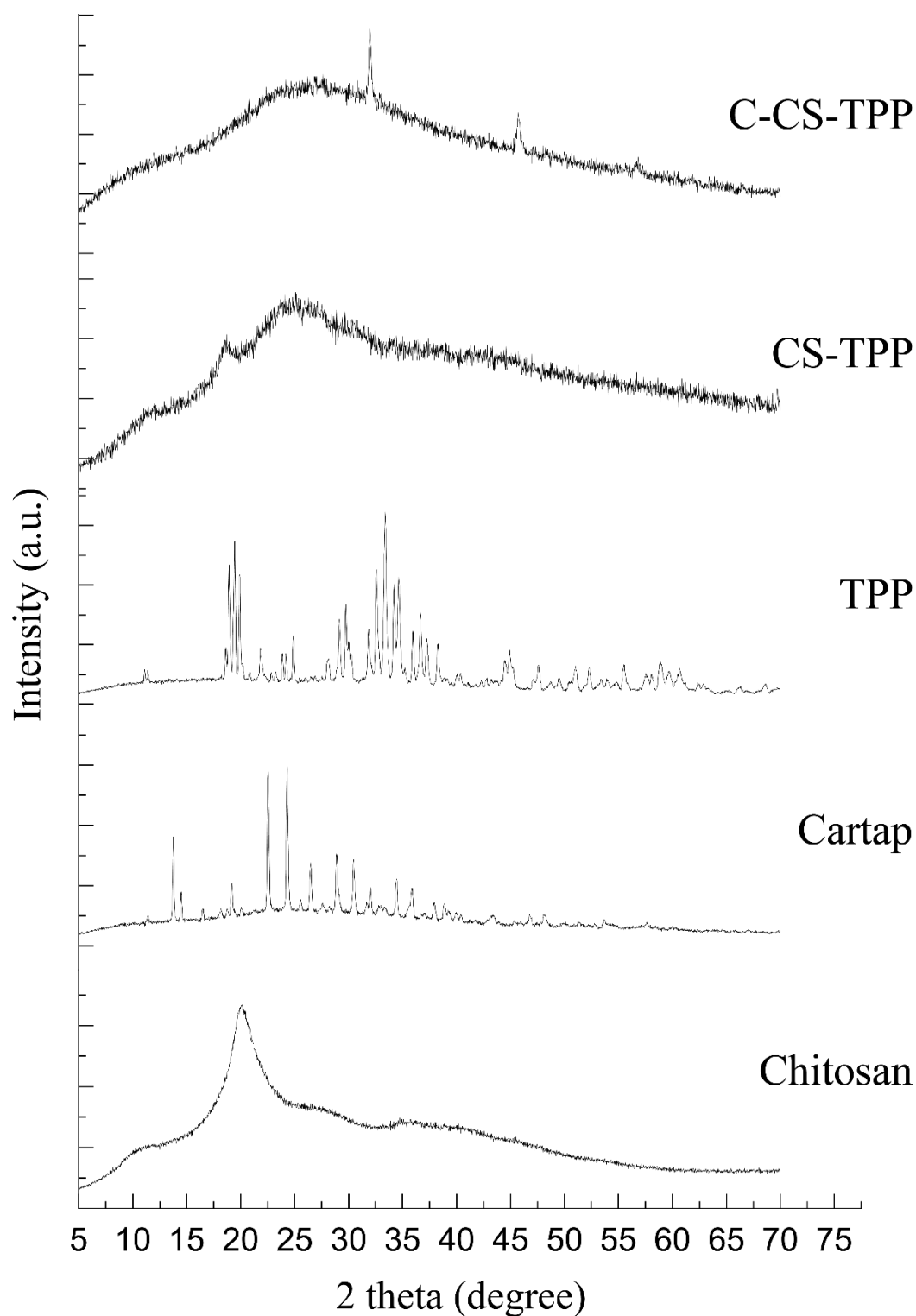


Fig. 4.41: XRD pattern of cartap, chitosan, sodium tripolyphosphate (TPP), chitosan nanospheres (CS-TPP) and cartap loaded chitosan nanospheres (C-CS-TPP)

Electrostatic interaction of cartap with chitosan and TPP acts as the main driving force for cartap loading in nanospheres (Mathew et al., 2010; Hao et al., 2002; Pendekal, 2013).

The intensity of characteristic peaks of chitosan was decreased in case on crosslinked chitosan nanospheres which were in accordance with study by Wang et al. (2001). Similar results were also reported when 5-fluorouracil/ leucovorins and pramipexole were encapsulated into chitosan nanoparticles by Papadimitriou et al. (2008) and Li et al. (2011) in their respective studies.

The X-ray diffraction pattern of cartap loaded C-CS-TPP nps showed peaks for both cartap and blank chitosan nanospheres which suggested that crystal structure of cartap remained the same in C-CS-TPP nps. Only the peak shifting and reduction in peak intensity was observed suggesting interaction between cartap and CS-TPP nps. This indicates that the conjugated and entrapped forms of the insecticide are unable to form their own crystal lattice inside the polymer and they are in amorphous state. These evidences lead to the conclusion that the insecticide was distributed over the nanostructure which satisfies the essential requirement of a good delivery system.

4.3.2 Chitosan alginate nanospheres (CS-ALG nps)

4.3.2.1 Synthesis of nanospheres

Chitosan nanospheres were synthesized through ionotropic gelification method (De and Robinson, 2003). Ionotropic gelation is based on the capability of polyelectrolytes to crosslink in the presence of counter ions (Giri, 2016). Alginate–chitosan nanospheres were prepared in a two-step procedure based on the ionotropic gelation of polyanion with calcium chloride followed by polycationic crosslinking. In acidic media, chitosan shows polycationic nature and represents $\text{-NH}^+ 3$ sites. Cationic sites in chitosan were responsible for the crosslinking of chitosan. In the first stage, cartap was mixed with alginate solution under continuous stirring and when calcium chloride was added, divalent ions (calcium in calcium chloride) replace the monovalent ions (sodium in sodium alginate) in the polymer. Thereafter, solution of polycation (chitosan) is added to obtain alginate nanospheres reinforced with polycation via polyelectrolyte complexation (Calija et al., 2017). The hetero atoms of cartap initiates formation of hydrogen bond with protonated chitosan chain to attain higher encapsulation. The mechanism of interaction in the formulation is given in Fig 4.42. Incorporating cartap hydrochloride onto the CS-ALG nanospheres exhibited a remarkable increase in their stability due to several electrostatic interactions between cartap and polymeric networks of chitosan. The amino groups of chitosan are responsible for interaction

with polyanions by means of inter and intramolecular cross linkages to form nanospheres (Xu and Du, 2003; Vila et al., 2004). It is only the free amino groups on which synthesis of nanospheres depends, as it increases the electrostatic interactions between the insecticide and the nanospheres to reduce the particle size (Yang and Hon, 2009; Janes et al., 2001).

4.3.2.2 Physicochemical characterization and stability of the nanoformulation.

Size distributions of the synthesized nanospheres were evaluated by dynamic light scattering. The average size of the blank nanospheres (CS-ALG) using the DLS was 214.6 ± 4.93 nm (Fig. 4.43a) but after incorporation of cartap, the size of loaded (C-CS-ALG) nps was increased to 381.77 ± 11.65 nm (Fig. 4.43b). Similarly, the polydispersity values for CS-ALG nps was 0.219 ± 0.003 and for insecticide loaded C-CS-ALG nps, it was 0.356 ± 0.12 . A PDI value <0.5 portrays that the suspension has homogenous size distribution. The zeta potential of CS-ALG nps was $+22.66 \pm 1.039$. However, cartap incorporation in the nanospheres resulted in increased stability, with higher zeta potential $+31.2 \pm 1.327$ mV in C-CS-ALG nps. The zeta potential value around ± 30 mV is assumed to be good for suspension (Kumar et al., 2014). DLS analysis of acetamiprid encapsulated chitosan alginate nps showed the size of nanocapsules 201.5 nm with a polydispersity index (PDI) of 0.390 and zeta potential value of -32.1 mV (Kumar et al., 2015). In a study by Maruyama et al, the average particle size of chitosan alginate nanospheres loaded with imazapyr and imazapic was 377.7 nm and zeta potential of -30 mV (Maruyama et al., 2016). Insecticide imidacloprid nanospheres was synthesized by using CS-ALG having size around 150 nm (Kumar et al., 2014). The hydrodynamic diameter of paraquat loaded chitosan nanospheres was around 635 nm having polydispersity value of 0.518 and zeta potential of -22.8 ± 2.3 mV (Dos et al., 2011).

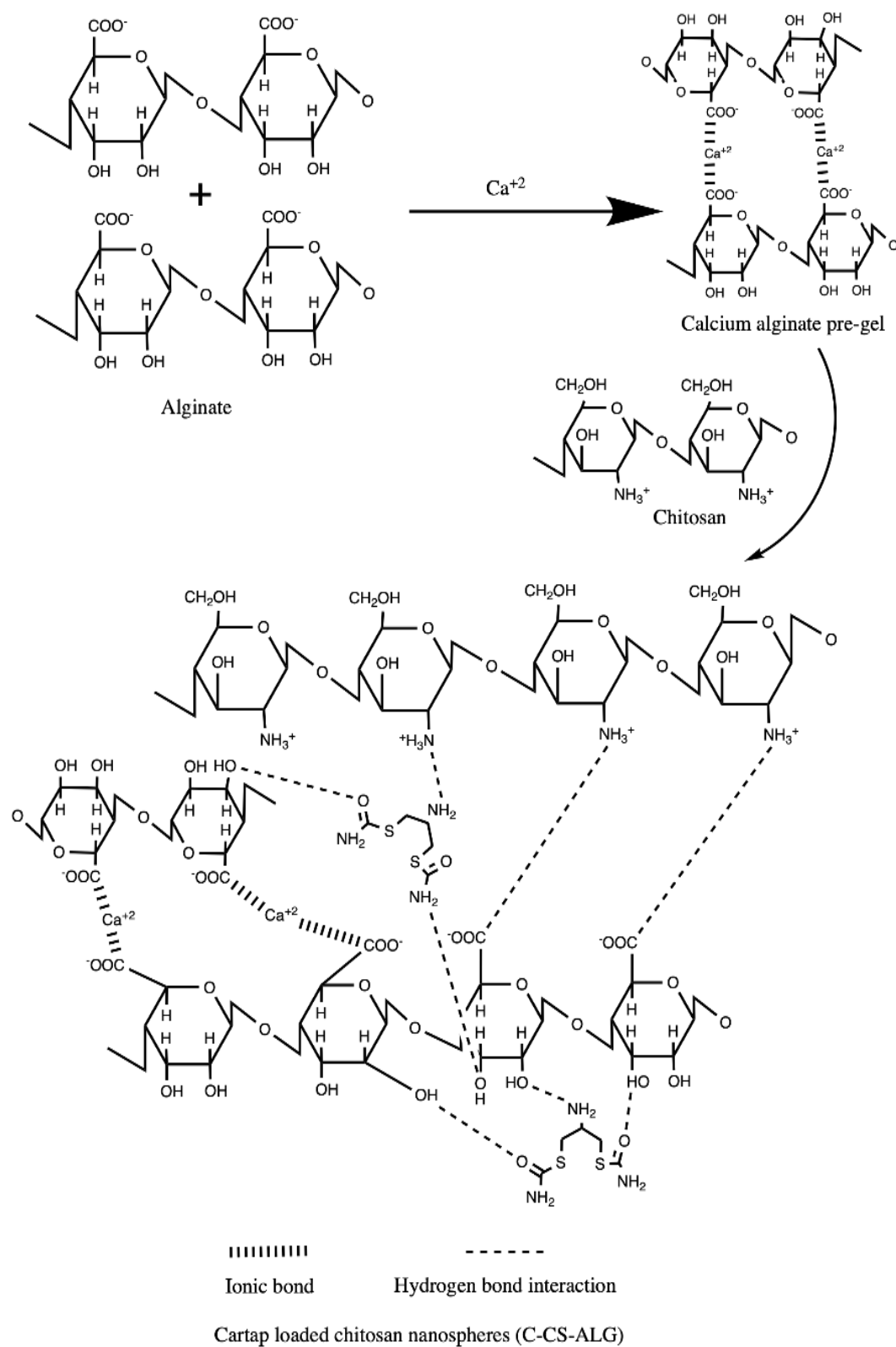


Fig.4.42: Possible mechanism of interaction of chitosan (CS), alginate (ALG) and cartap hydrochloride (C) to synthesize insecticide loaded chitosan nanospheres (C-CS-ALG)

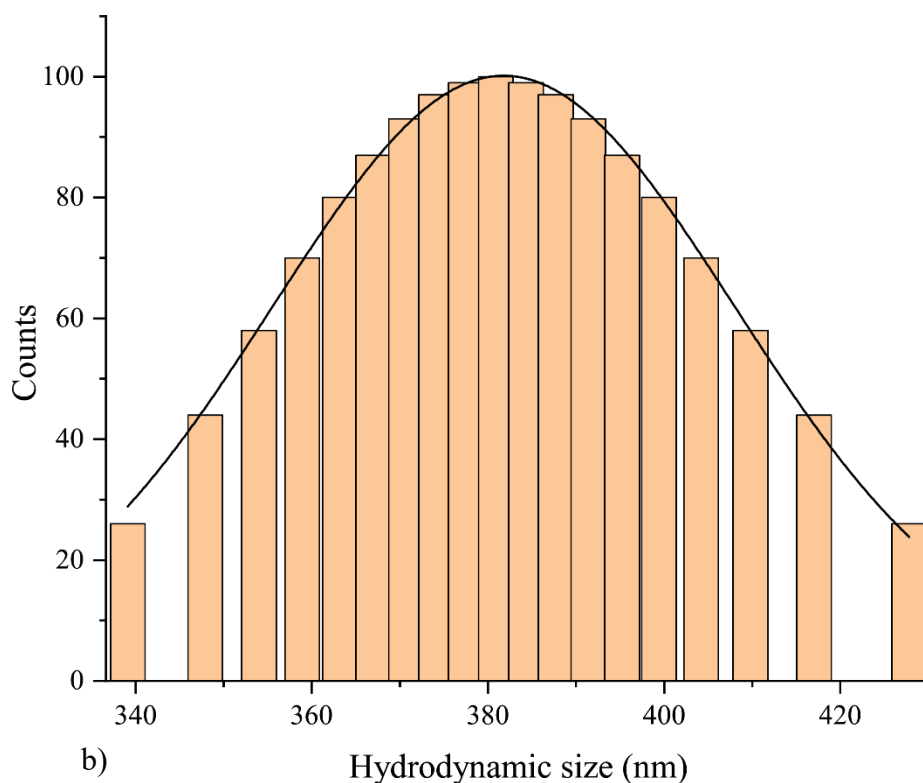
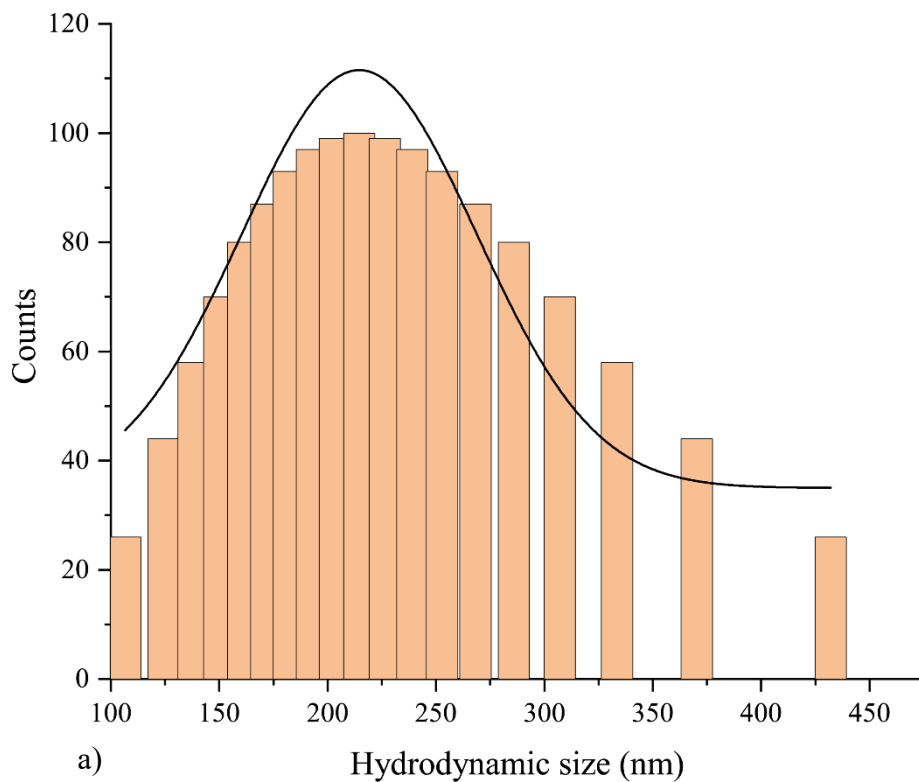
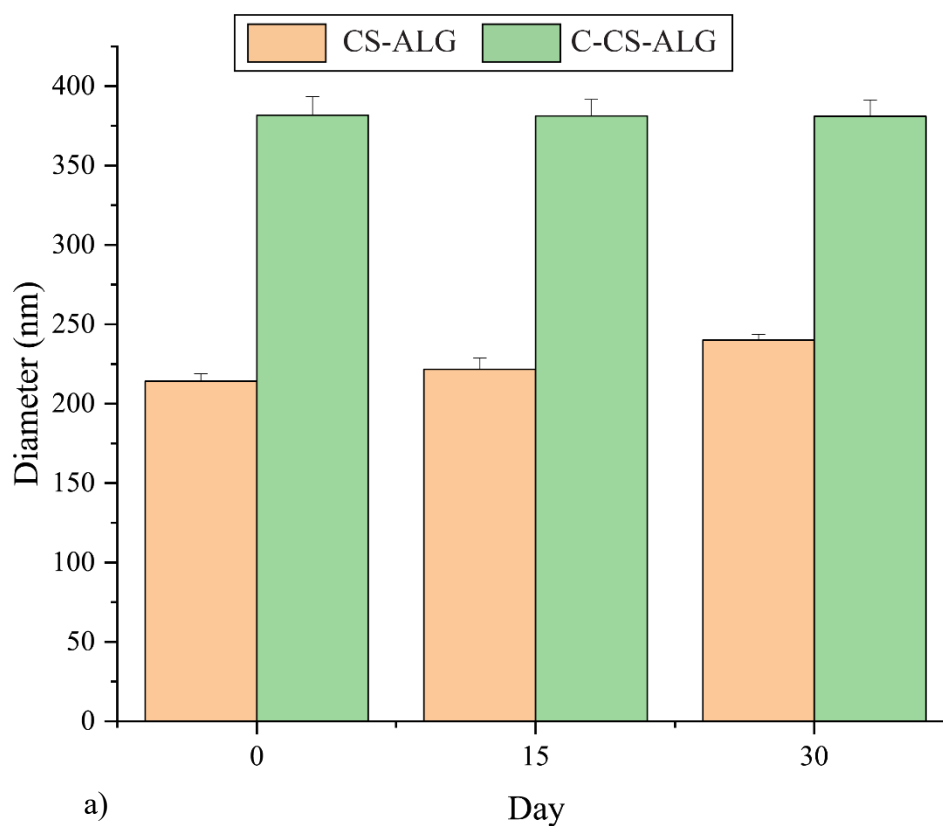


Fig. 4.43: Average size distribution analysis of a) chitosan alginate nanospheres (CS-ALG); b) cartap loaded chitosan nanospheres (C-CS-ALG) by dynamic light scattering (DLS).

The stability of the synthesized nanospheres was assessed by evaluating zeta potential, polydispersity and average size over the duration of 30 days (Fig. 4.44). The particles

remained stable in the presence of insecticide with almost no significant change in above parameters. The average size of cartap loaded nanospheres after 30 days was 378.9 ± 10.39 nm with polydispersity value 0.327 ± 0.004 . The zeta potential was also maintained at around 32.4 ± 1.47 mV, implying that electrostatic repulsion between the particles was maintained thereby decreasing the probability of aggregation. The average size, zeta potential, and polydispersity values of blank chitosan nanospheres was 233.4 ± 3.6 , 0.257 ± 0.004 and 24 ± 0.693 respectively, suggesting particle aggregation while insecticide incorporation made the particles more stable by increasing their zeta potential. This could be due to the interactions between the cartap and polymeric chains of the chitosan nanospheres. During this period, blank chitosan nanospheres showed higher polydispersity values than cartap incorporated nanospheres. In a study by Silva et al, it was reported that chitosan nanoparticles loaded with paraquat was stable for 60 days at ambient temperature (Dos et al., 2011). As compared to blank nanoparticles, stability of imazapyr and imazapic encapsulated chitosan nanoparticles were increased in terms of average size, polydispersity and zeta over period of 30 days (Maruyama et al., 2016).



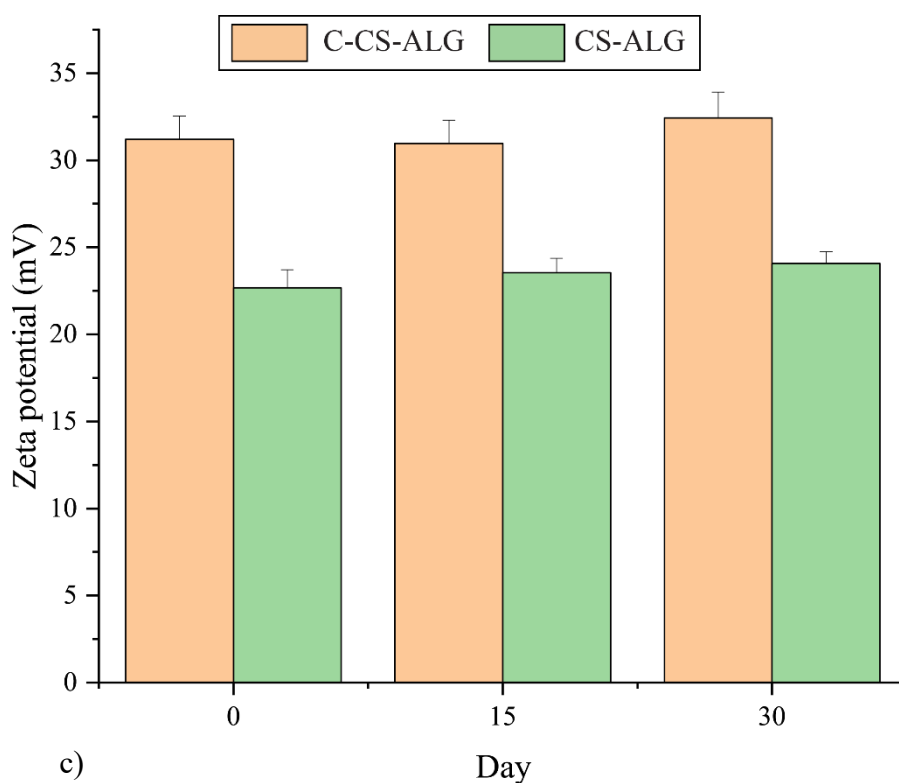
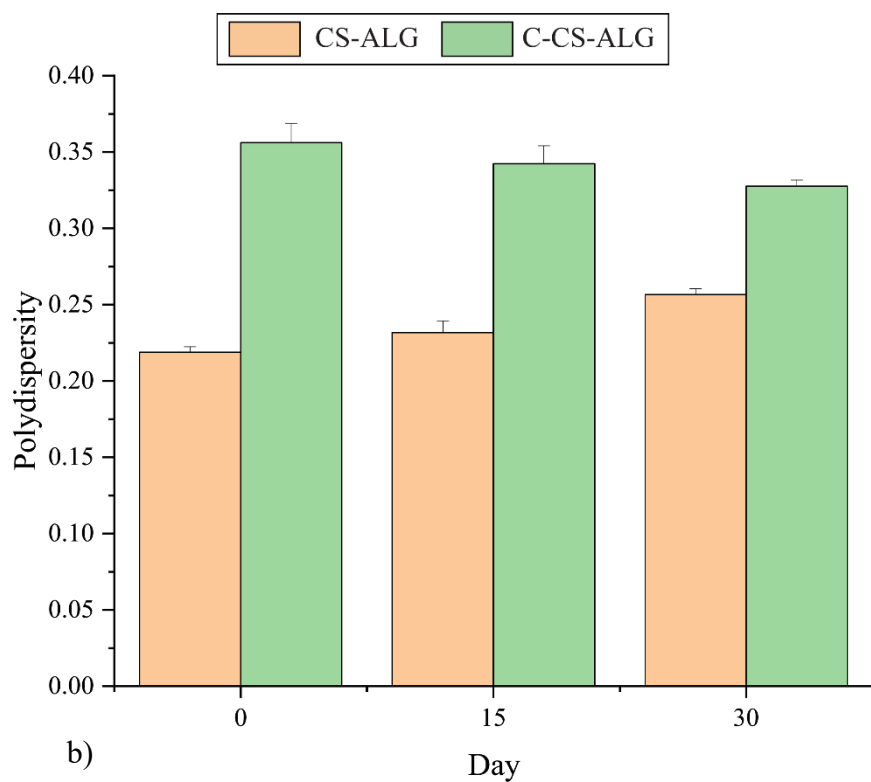


Fig. 4.44: Stability of nanoparticles over a period of 30 days. CS-ALG: chitosan alginate nanospheres; C-CS-ALG: cartap chitosan alginate nanospheres a) Average size b) Polydispersity c) Zeta potential

4.3.2.2.1 FTIR

FTIR studies of chitosan, sodium alginate, cartap, chitosan nanospheres and cartap loaded chitosan nanospheres were performed to have a deeper understanding of the mechanism of nanoparticle formation and to recognize interactions between them (Fig. 4.45, 4.46). The characteristic peaks of the chitosan were shown at 3396.5, 2908, 2879.4, 2166.8, 1663.4, 1381, 1074.2 and 1028.4 cm^{-1} . The stretching vibration of $-\text{OH}$, the extension vibration of $>\text{NH}$, and the intermolecular hydrogen bonds of the polysaccharide were ascribed to the broad peak around 3600–3000 cm^{-1} in pure chitosan. Intense broad peak at 3396.5 cm^{-1} indicate OH stretching and NH stretching vibration (Baysal et al., 2013). The bands at 2908 and 2874 cm^{-1} represented symmetric and antisymmetric CH stretching modes of vibration. The absorbance peak at 1154.5 cm^{-1} reflects the amino groups of chitosan (Dos et al., 2011). The amide I, II and III bands at 1663 cm^{-1} , 1594.6 cm^{-1} , 1320.6 cm^{-1} (Pawlak and Mucha, 2003; Dong et al., 2002) indicates that chitosan had a high degree of deacetylation. The bands between 960 to 1180 cm^{-1} corresponds to saccharide peaks of chitosan. The band near 1074.2 cm^{-1} represented C-O-C asymmetric stretch.

The absorption spectrum of the cartap molecule (Fig. 4.46) showed from which the main bands can be seen at 1670.5 cm^{-1} corresponding to C O stretching, 3309.9 cm^{-1} and 3239 cm^{-1} were associated with NH stretching, 2978.9 cm^{-1} was due to NCH_3 stretching and 1188.6 to 1024.8 cm^{-1} confirms CS stretching (Rahim et al., 2018).

FTIR spectra of sodium alginate showed band at 860.4 cm^{-1} (mannuronic acid), 947.6 cm^{-1} (uronic acid), 3200-3400 cm^{-1} (OH group) and 2930.5 cm^{-1} (CH_2 stretching) (Aprilliza, 2017). Peaks near 1623.2 cm^{-1} represents symmetric COO^- stretching vibration and 1416.4 cm^{-1} are associated with the asymmetric COO^- stretching vibration (Dos et al., 2011). Both these peaks correspond to carbonyl groups of alginate.

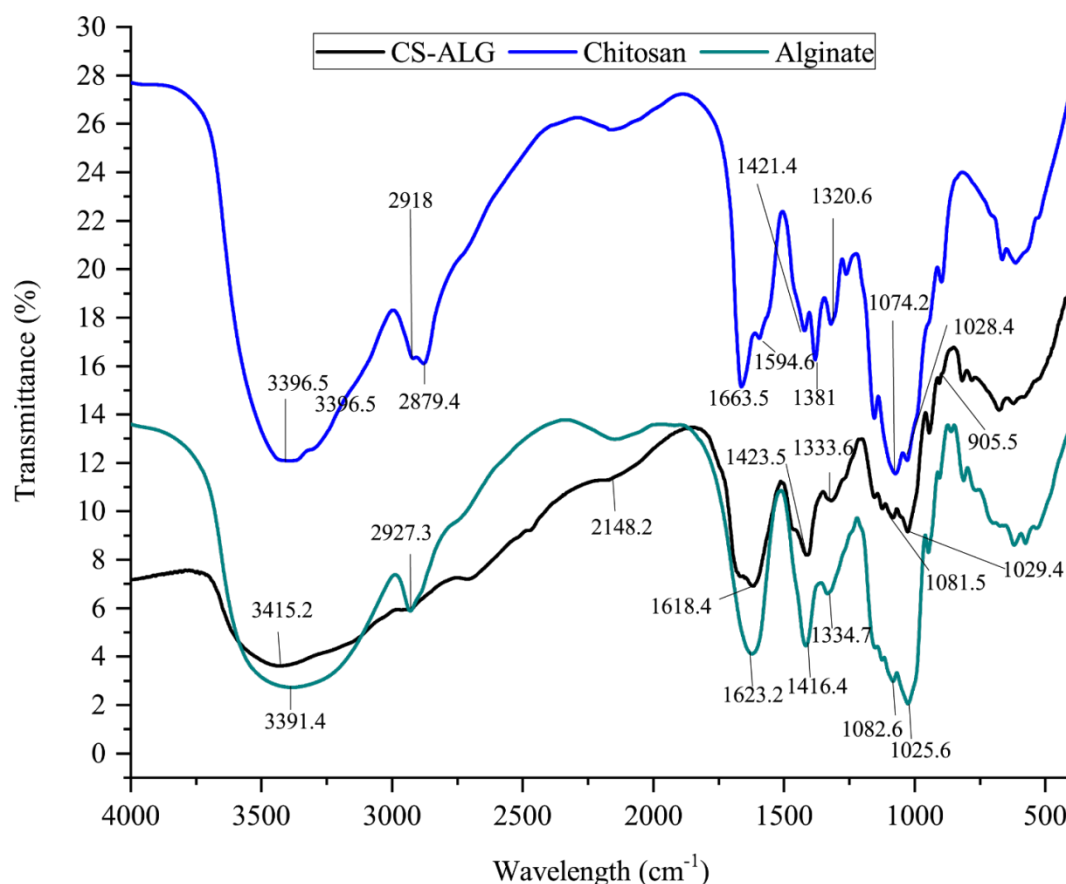


Fig. 4.45: FTIR spectrum of chitosan (CS), sodium alginate (ALG) and chitosan nanospheres (CS-ALG)

Blank chitosan nanospheres showed absorption bands at 3415.2, 2927.3, 2152.9, 1618.4, 1423.5, 1383.5 and 1029.4 cm^{-1} respectively (Fig. 4.45). The broad absorption band at 3415.2 cm^{-1} represents OH stretching vibration. OH bonding in nanospheres was confirmed by the bands in the spectral region between 3200 and 3550 cm^{-1} . The band at 2927.3 cm^{-1} and 1618.4 cm^{-1} confirmed asymmetric stretching vibration of methylene and NH bending vibration respectively (Dong et al., 2002; Hashad et al., 2016). The band for N–O stretching at 1383.5 cm^{-1} confirms stretching of aliphatic nitro compounds (Krajewska, 2005). The band at 1663.4 cm^{-1} was shifted to 1618.4 cm^{-1} in case of nanospheres confirmed CO stretching of chitosan, indicating the interaction between nanospheres (Jayakumar et al., 2011). The band at 1029.4 cm^{-1} confirmed CO stretching vibration of primary alcohol.

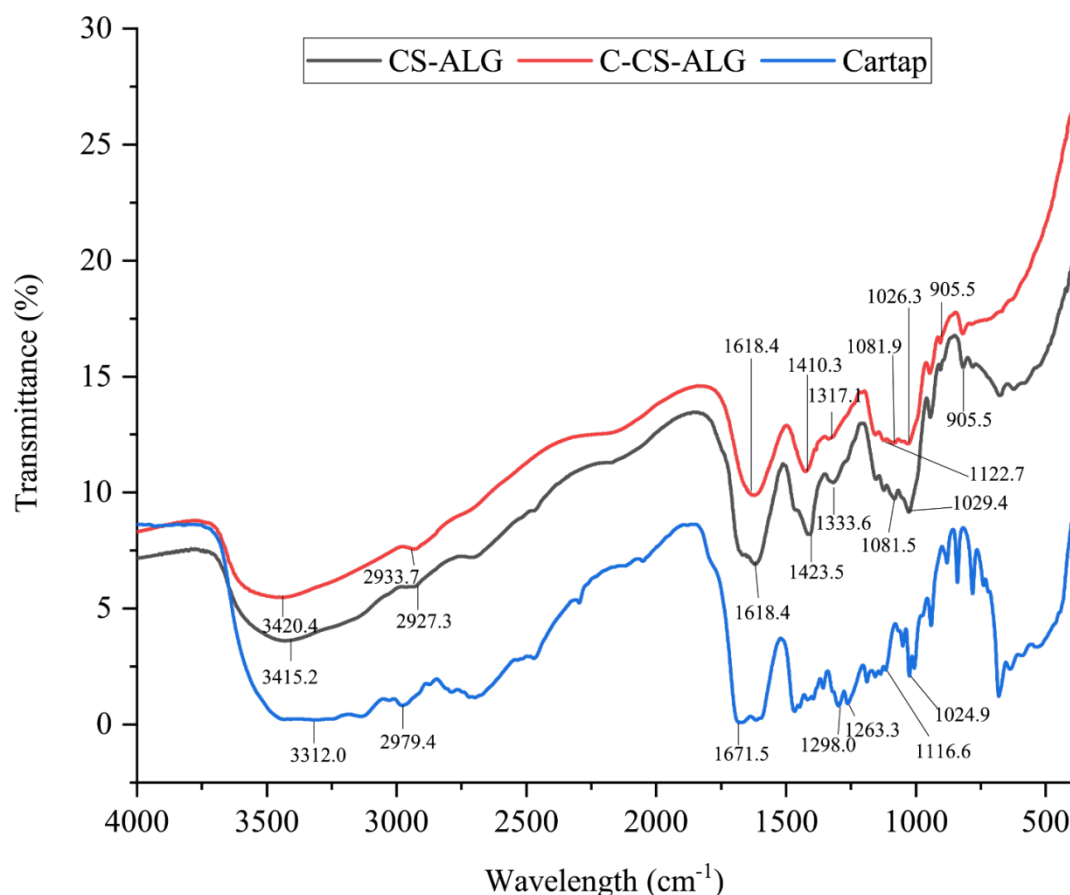


Fig. 4.46: FTIR spectrum of cartap, chitosan nanospheres (CS-ALG) and cartap loaded chitosan nanospheres (C-CS-ALG)

The FTIR spectrum of CS-ALG nps and C-CS-ALG nps (Fig. 4.46) were consistent with previously published data (Kumar et al., 2014). The comparative spectra, in case of CS –ALG nanospheres, are presented in the areas of hydroxyl and carbonyl groups absorptions, where several shifts of the characteristic peaks were observed. In case of blank nanospheres, presence of broad band at 3415.2 cm^{-1} confirms O H and N H stretching modes but this band got shifted to 3420.4 cm^{-1} in cartap chitosan nanospheres suggesting the existence of hydrogen bond interactions (Xu and Du, 2003; Qin et al., 2006; Dudhani et al., 2010; Wu et al., 20005; Gomathi et al., 2017; Lazaridou et al., 2020). These shifts are clearly due to the ionic interaction taking place between CS and alginate for the formation of nanospheres. The peak shifting from 1663.4 cm^{-1} to 1618.4 cm^{-1} for both blank and insecticide loaded nanospheres confirmed non-bonding interactions of CO groups (Rejinold et al., 2011). Cartap also represented two peaks in these areas as at 1670.5 and 1615.8 cm^{-1} . It is characteristic that in all C-CS-ALG nanospheres these peaks were not recorded and it seemed that the lower intensity peak at 1615.8 cm^{-1} was shifted to slightly higher wavenumbers and merged with the carbonyl absorption of CS-ALG forming a new peak with absorption at 1618.4 cm^{-1} . This

finding indicates that the carbonyl peaks of cartap also participated in hydrogen bond interactions with CS (Lazaridou et al., 2020).

A comparison between FTIR spectra of chitosan, chitosan nanospheres and cartap loaded chitosan nanospheres (Table: 4.2) revealed that the peak at 1594.6 cm^{-1} (C N stretching vibration peak) disappeared when chitosan nanospheres were formed while all other characteristics peaks were present (Fig. 4.46) (Rahim et al., 2018). In chitosan nanospheres the broad band from 1623.2 cm^{-1} got shifted to 1618.4 cm^{-1} , and from 1416.4 to 1423.5 cm^{-1} after complexation of alginate with the chitosan polymer, (Maruyama et al., 2016) while in case of cartap loaded nanospheres, these bands shifted to 1618.4 and 1410.3 cm^{-1} (Dos et al., 2011).

An ionic interaction between the carbonyl group of alginate and the amino group of chitosan is responsible for observed alterations in the absorption bands of amino groups, carboxyl groups, and amide bonds (Ribeiro et al., 2005). Furthermore, after complexation, the band for amino group of chitosan was present at 1153 cm^{-1} , whereas it was absent in alginate. Similar observations were noted previously (Mitrevej et al., 2001). These results suggest an effective interaction between polymers and seem to be in agreement with the stoichiometric ratios between them indicating a prevalence of alginate and cartap in the final blend.

Table 4.2: Characteristic wavenumber of chitosan (CS), cartap hydrochloride (CH), chitosan nanoparticles (CS-ALG) and cartap incorporated chitosan nanoparticles (C-CS-ALG)

Functional groups	CH	CS	CS -ALG	C-CS-ALG
–OH–	3431	–	3429	3419
	630		602	602
–CO ₃ ^{2–} –	–	897.5	818.2	816.9
–OH stretching, –OH hydrogen-bonded, NH ₂ asymmetric stretching, N–H stretching in inter chain NH–O– C	3309.9 3239	3396.5	3415.2	3420.4
–CH ₂ – asymmetric	2978.9	2908	2927.3	2933.7
		2879.4	Disappear	2703.8
C=O stretching. (1650-1590)	1670.5	1663.4	1618.4	1618.4
Deformation δ N–H and C–N (Amide II)	–	1594.6	Disappear	Disappear
Deformation –OH in C–O–H	–	1421	1423.5	1410.3
C–N stretching coupled with NH in plane deformation (amide III band)	–	1381	1333.6	1317.1
CH ₂ wagging coupled with OH in plane deformation	1357	1320.6	1333.6	1317.1
CS stretching	1188.6- 1024.8	–	Disappear	1122.7
C–N stretching of the amine (1220-1020)	–	1154.5	1154	1156
C–O stretching (–CH–OH, the secondary hydroxyl group)	–	1074.2	1081.5	1081.9
C–O stretch (–CH ₂ –OH the primary hydroxyl group)	1024.8	1028.4	1029.4	1026.3
Deformation N–H in primary amine	– 941.1 679.8	897.5 945 665.3	905.5 945.8 674	905.5 943.9 674.7

4.3.2.2.2 FESEM

FESEM micrographs revealed that both blank and insecticide loaded nanospheres were spherical in shape and in nano size range (Fig. 4.47). The size of blank nanospheres ranged from 101.66 to 158.33 nm (Fig. 4.47a), while insecticide loaded chitosan nanospheres measured between 107.58 and 173.07 nm (Fig. 4.47b). The size of the cartap loaded

nanospheres increased after encapsulation, as evidenced by FESEM pictures and these results were corroborated by DLS study.

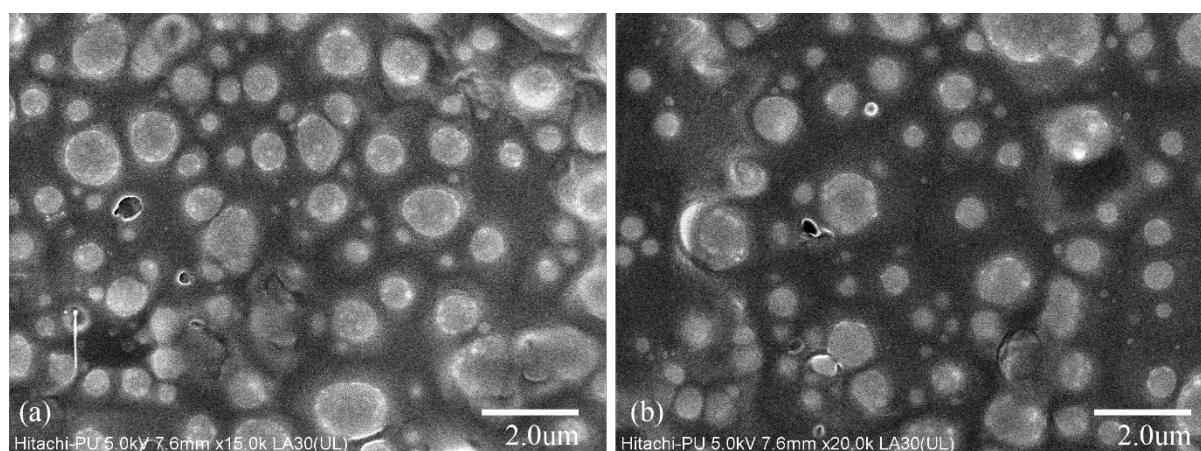


Fig. 4.47: FESEM micrographs of a) chitosan nanospheres (CS-ALG); b) cartap loaded chitosan nanospheres (C-CS-ALG)

4.3.2.2.3 XRD

X ray diffractogram of chitosan, cartap, sodium alginate (ALG), blank chitosan nanospheres (CS-ALG) and cartap loaded chitosan nanospheres (C-CS-ALG) were illustrated in Fig.4.48. Sodium alginate is usually crystalline due to strong interaction between the alginate chains through intermolecular hydrogen bonding (Venkatesan et al., 2017). Sodium alginate, showed distinctive peaks from 10 to 60° at a diffraction angle of $2\theta \approx 14.76^\circ, 18.43^\circ, 22.77^\circ, 25.52^\circ, 30.22^\circ, 34.42^\circ, 33.77^\circ, 45.90^\circ$ which confirmed its crystalline nature due to the reflection of their (110) plane from poly guluronate unit, (200) plane from poly mannuronate and the other from amorphous halo (Pendekal and Tegginamat, 2013; Aprilliza, 2017). Crystalline nature of chitosan was proved by sharp diffraction peak near $2\theta \approx 20^\circ$ (Fang et al., 2011). In case of cartap hydrochloride, sharp peaks were detected at $2\theta \approx 11.3^\circ, 13.7^\circ, 14.5^\circ, 16.4^\circ, 19.0^\circ, 22.5^\circ, 24.3^\circ, 26.5^\circ, 28.8^\circ, 30.5^\circ, 31.9^\circ, 34.3^\circ, 35.9^\circ, 37.9^\circ, 38.9^\circ, 45.9^\circ, 46.8^\circ$ and 48.2° confirming its crystalline nature. Diffraction peaks at $2\theta \approx 24.08^\circ, 31.8^\circ$ and 45.8° were observed for cartap incorporated chitosan nanospheres (C-CS-ALG) nps.

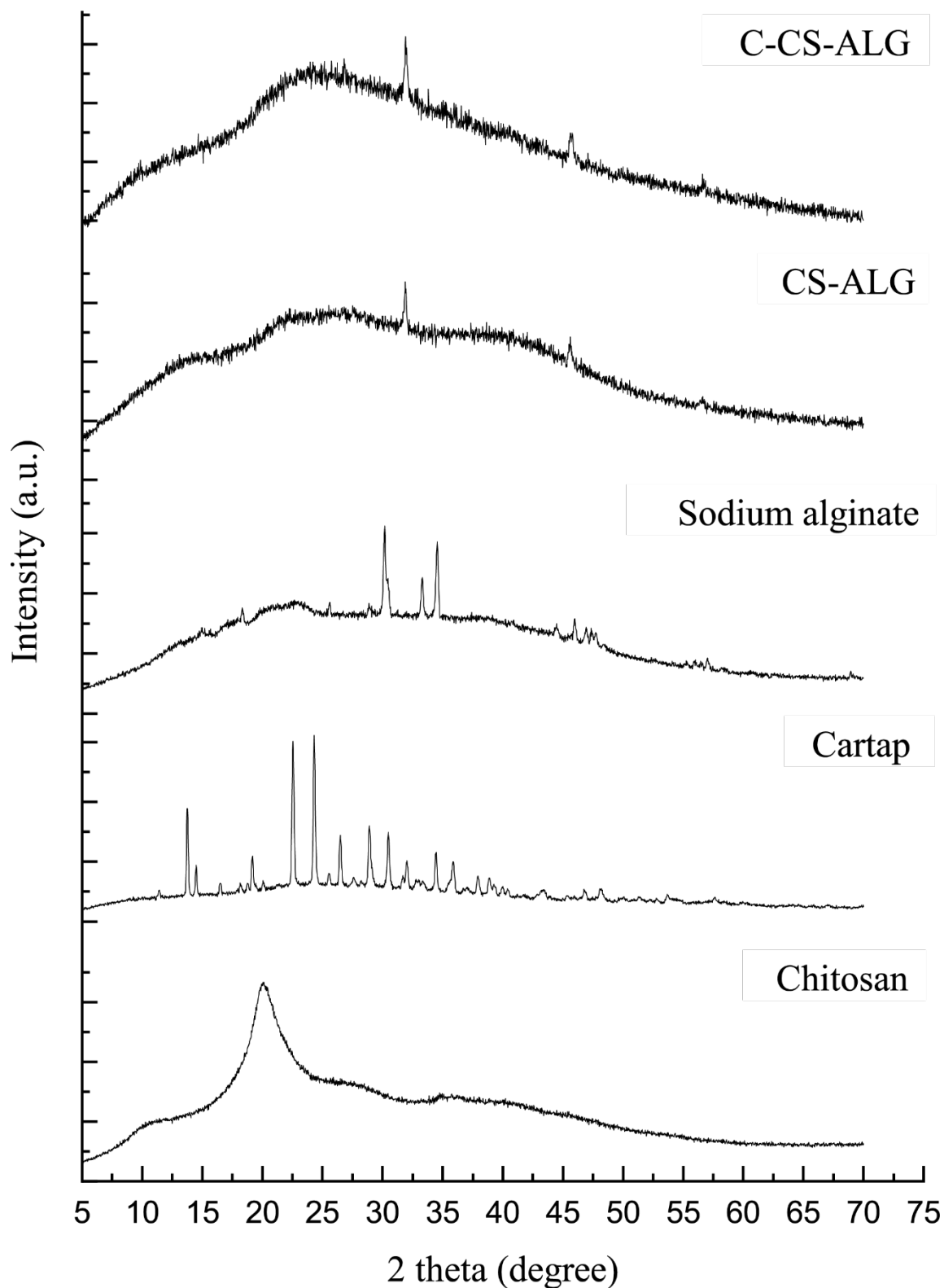


Fig. 4.48: XRD diffractogram of cartap, chitosan (CS), sodium alginate (ALG), chitosan nanospheres (CS-ALG) and cartap loaded chitosan nanospheres (C-CS-ALG)

After complexation, the characteristic peaks of chitosan faded and an amorphous morphology was observed in the nanospheres. The amorphous character of cartap loaded chitosan nanospheres was confirmed by the widening of diffraction peaks at $2\theta \approx 24.08^\circ$. The

complex formation between sodium alginate and chitosan altered the crystalline structure of chitosan, which resulted in the elimination of hydrogen bonding between hydroxyl groups and amino groups in chitosan (Fabia et al., 2005). Due to this molecular arrangement in the crystal lattice, different diffraction peaks for chitosan and cross-linked chitosan were observed.

The intensity of characteristic chitosan peaks was decreased during synthesis of nanoparticles which correlates with the study by Aprilliza (2017). Maity et al. (2017) and Fang et al. (2011) reported similar findings when naringenin and silver nanoparticles were encapsulated into chitosan nanoparticles in their respective studies.

X-ray diffractogram of insecticide loaded nanospheres revealed peaks for both blank nanospheres and cartap, indicating reduction in peak intensity and peak shifting, confirming the interaction between insecticide and chitosan nanospheres. This implies that the entrapped and conjugated forms of the cartap seems unable to build their own crystal lattice within the polymer and therefore existed as amorphous compound. These findings suggest that the insecticide was dispersed throughout the nanostructure, meeting the main criteria of a good delivery system.

4.4 Evaluation of release kinetics nano-cartap hydrochloride

4.4.1 Encapsulation efficiency of C-CS-TPP nps

The encapsulation efficiency (EE) of chitosan nanospheres during loading of cartap was determined through indirect measurements using UV-spectrophotometer at wavelength of 215 nm. About 86.05% of entrapment efficiency was found for cartap loaded nanospheres. Cintia et al. (2016) developed nanoparticles of chitosan with TPP for encapsulation of imazapic and imazapyr which showed an encapsulation efficiency of 50 to 70%. The EE value of insecticide nicotine hydrochloride nps was approximately 55% (Yang et al., 2018). The encapsulation efficiency obtained for the CS-TPP nanoparticle formulation containing Paraquat was 62.66%, indicating good affinity between the components (Grillo et al., 2014a). The encapsulation efficiency of chitosan nanoparticles for hexaconazole was 73% (Chauhan et al., 2017).

4.4.2 Encapsulation efficiency C-CS-ALG nps

The encapsulation efficiency (EE) of cartap loaded chitosan nanospheres was determined spectrophotometrically through indirect measurements at wavelength of 215 nm. Cartap loaded chitosan nanospheres had an entrapment efficiency of 76.19%. Chitosan alginate nanospheres of imazapic and imazapyr offered encapsulation efficiency of 62.3 and 71.3% respectively as explained by Maruyama et al. (2016). 74.2% encapsulation efficiency was obtained when paraquat was incorporated in chitosan alginate nanoparticle indicating good affinity between the components (Dos et al., 2011). Similarly, in a study by Silva et al, 60% encapsulation was reported for the herbicides clomazone (Silva et al., 2010).

4.4.3 Release mechanism of cartap from chitosan tripolyphosphate nanospheres

The *in vitro* release kinetics of nanospheres offers crucial details about their ability to alter their release profile, and is therefore an important parameter to consider when evaluating the nanoparticle stability, effectiveness, and efficiency. The cumulative release percentage of cartap from nanoformulations was calculated and found that the maximum time of release for the nanoformulation was 15 days (Fig. 4.49). Within 6 days more than half of the cartap was released from the formulation. Furthermore, a controlled release pattern was also found for the nanoformulation. The release kinetics of insecticide from encapsulated nanospheres was studied by employing five different mathematical models: zero order, first order Higuchi, Korsmeyer-Peppas and Hixson. Fig. 4.49- 4.54 shows the results obtained from linearization of the release curves for encapsulated cartap. The values of release exponent and release constant were calculated by applying linear regression (Table 4.3).

Table 4.3: Parameter values obtained by fitting of mathematical models to the data for the chitosan tpp nanospheres containing insecticide

	Release constant (k) min ⁻¹	Release exponent (n)	Correlation coefficient (r)
Zero order	27.12	0.133	0.813
First order	1.807	-0.001	0.962
Hixson	0.43	0.003	0.927
Higuchi	11.27	3.556	0.944
Peppas	0.508	0.576	0.764

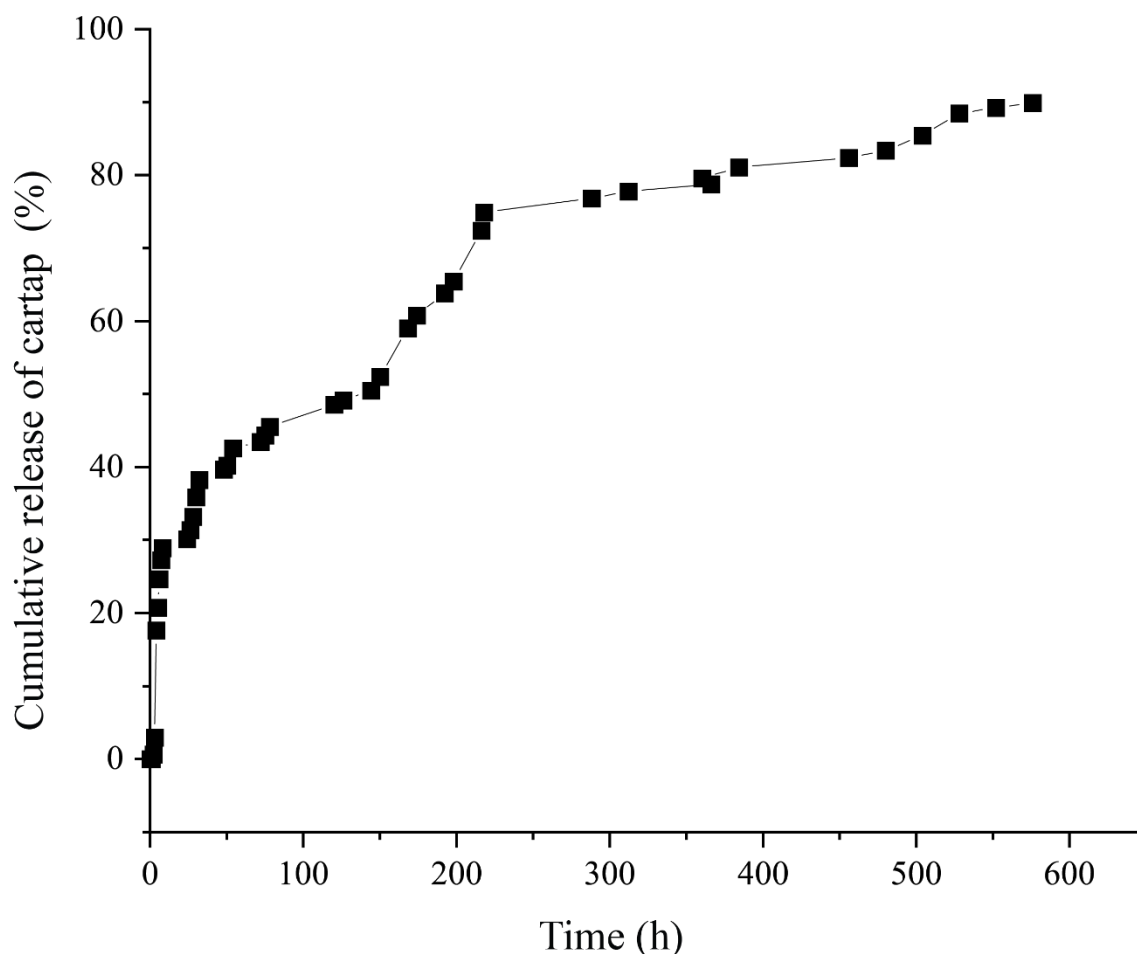


Fig. 4.49: *In vitro* release kinetics profile of cartap loaded nanospheres

In Zero order kinetics, insecticide release kinetics was studied by plotting cumulative insecticide release vs. time and from the slope of plot (Agnihotri et al., 2012), it can be concluded that cartap release from the matrix does not follow zero-order release kinetics as r^2 was approaching 0.81 (Fig. 4.50). First order release follows linear kinetics and cartap release from in-vitro dissolution study was plotted against time i.e., log % of insecticide remaining vs. time and the value of correlation coefficient $r^2 = 0.96$ (Fig. 4.51) were calculated from slope of the plot which justified that insecticide release from the matrix follow the principle of first order release kinetics (Agnihotri et al., 2012).

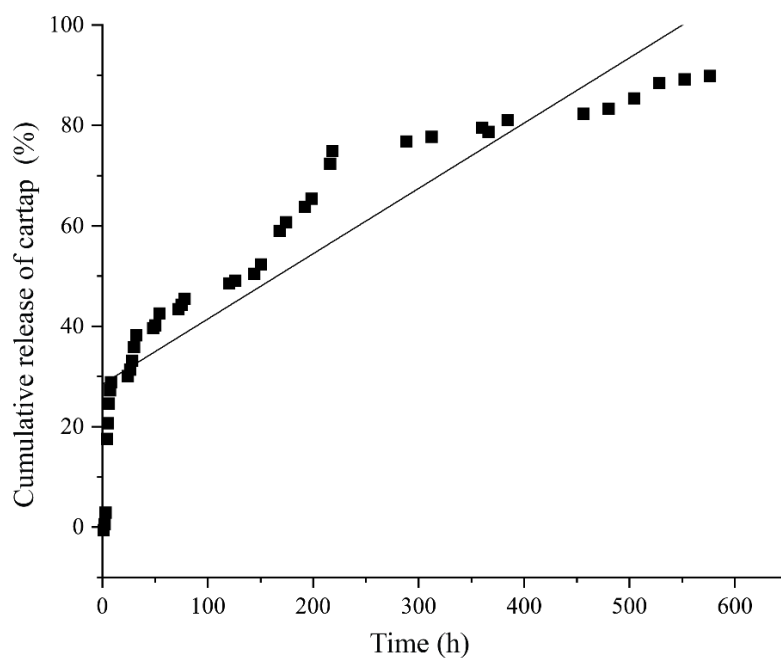


Fig. 4.50: Analysis of cartap release from nanospheres using zero order release kinetics

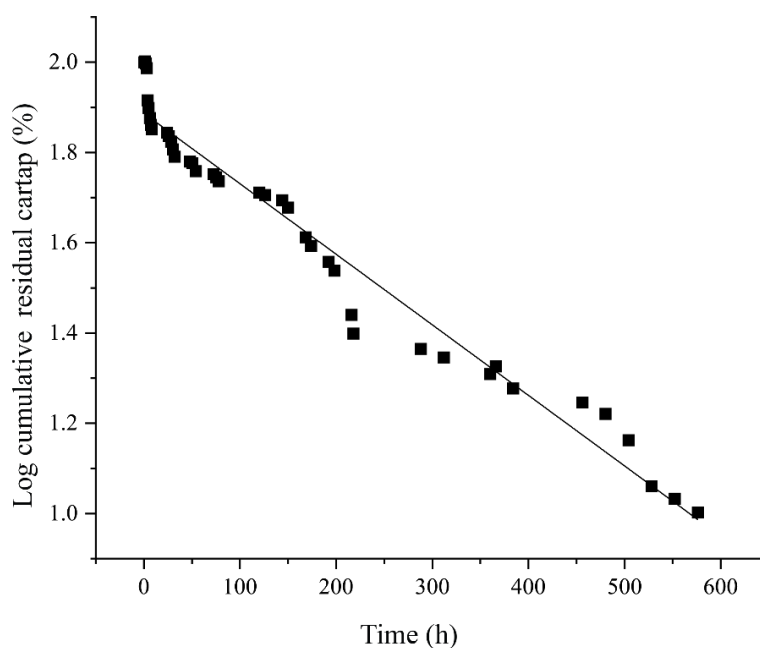


Fig. 4.51: Analysis of cartap release from nanospheres using first order release kinetics

Higuchi model is the best-known model to study release kinetics from controlled release systems, as it involves both dissolution and diffusion. The plot was generated between cumulative percentage insecticide versus square root of time and slope of plot (correlation coefficient $r^2 = 0.94$) (Fig. 4.52) represented that cartap release from the matrix followed Higuchi release model (Dash et al., 2010). The Hixson-Crowell cube root law defines the release from systems where there is a change in diameter and surface area of particles. graph

was plotted between cube root of insecticide percentage remaining in matrix versus time to study release kinetics of insecticide and the value of correlation coefficient ($r^2 = 0.92$) (Fig. 4.53) showed that change in surface area and diameter of the nanospheres with the progressive dissolution had slight effect of release (Subal., 2006). The release of cartap from nanospheres obeyed Hixson Higuchi and first order kinetics with goodness of fit ranked in order: first-order > Higuchi > Hixson-Crowell.

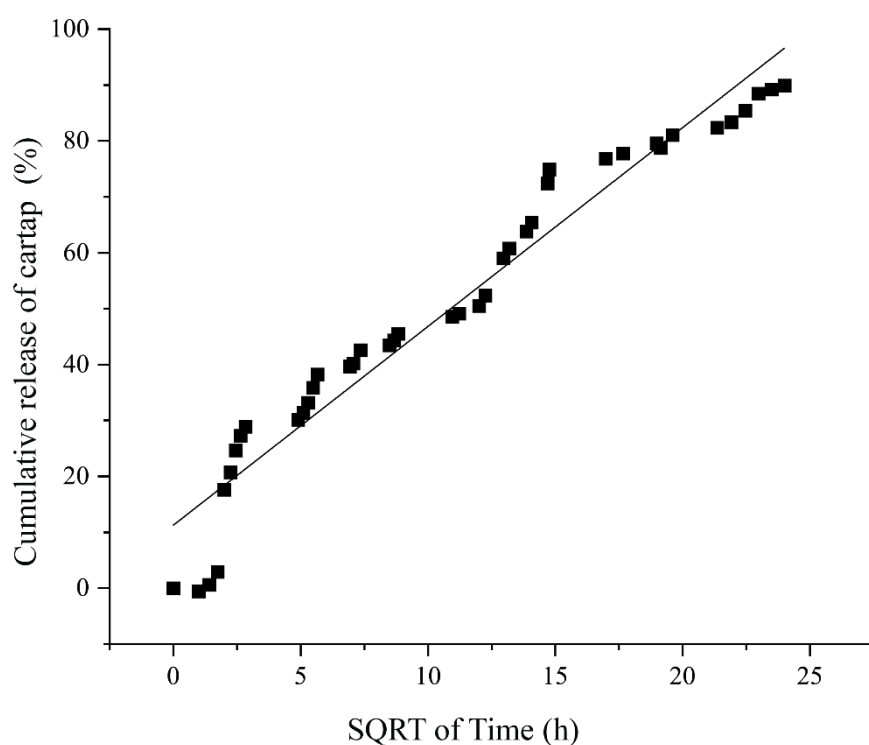


Fig. 4.52: Kinetic analysis of cartap release from nanospheres using Higuchi model

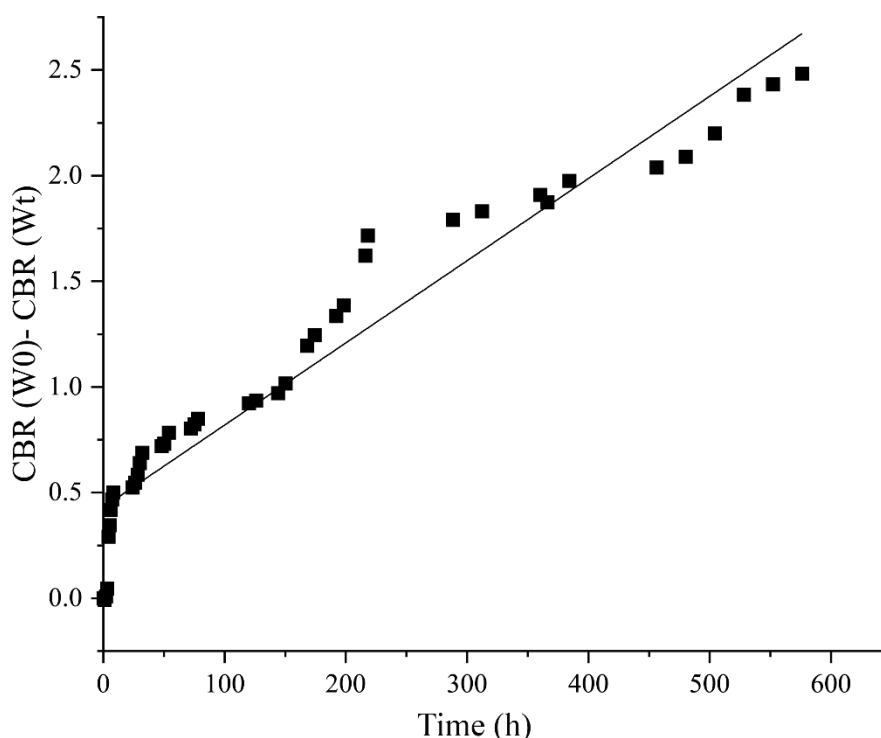


Fig. 4.53: Kinetic analysis of cartap release from nanospheres using Hixson Crowell model

When it's verified that insecticide was released by diffusion, the next step is to figure out the form of diffusion. To understand this, Korsmeyer and Peppas model was used to fit the release data (cumulative % drug release vs. log time) which defines that what type of diffusion was followed by insecticide from system and n value is used to characterize different release mechanisms. Diffusion constant (n) is an important marker for the mechanism of active ingredient release from the nanospheres. The value of n defines Fickian and non-Fickian behaviors. Case I transport: $n \leq 0.43$ states a Fickian release, which means the process is majorly regulated by diffusion. Case II transport: $n = 0.85$ implies a process that is defined by relaxation of the polymer. Intermediate values of n , i.e., $0.43 < n < 0.85$, indicate a non-Fickian release in which both diffusion and relaxation occur concurrently (Singhvi and Singh, 2011; Ritger and Peppas, 1987). The value of release coefficient by using Korsmeyer-Peppas mathematical model was 0.57 (Fig. 4.54) which suggested that release of cartap was by means of non-Fickian diffusion mechanism as its values lie between $0.43 < n < 0.85$. The value ($n = 0.57$) proved the combination of diffusion and erosion behavior. The mechanism was controlled by the chain relaxation process of chitosan. The dispersing medium penetrates into the system, forms pore and then erodes it. The erosion may also be the charge neutralization of chitosan amine groups and OH^- of buffer medium at alkaline pH. Due to this

charge neutralization, the release process of active ingredient was slower (Singhvi and Singh, 2011).

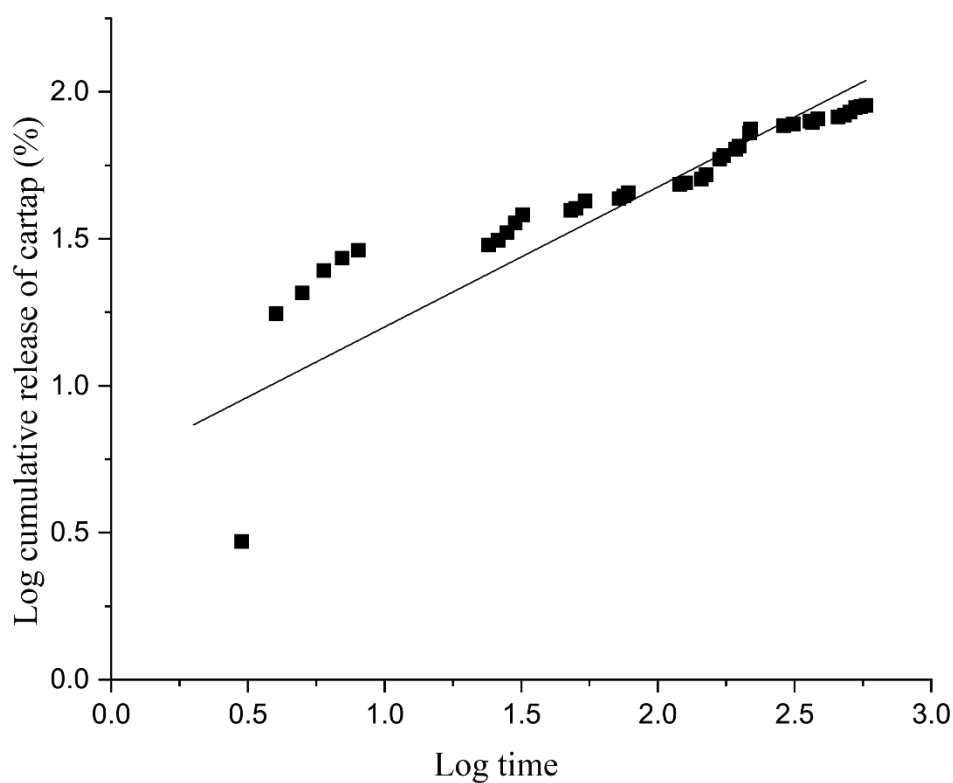


Fig. 4.54: Kinetic analysis of cartap release from nanospheres using Korsmeyer Peppas model

In the similar study by Silva et al, significant differences were observed between the release profiles of encapsulated and free paraquat (Silva et al., 2011). Maruyama et al, showed the release of imazapyr and imazapic was by diffusion following Fick's law, described by the Higuchi model and the release of IMR obeyed the Korsmeyer-Peppas mathematical model and indicated release by particle dissolution (Cintia et al., 2016). Modification in the release profile of paraquat and atrazine was observed when they get coated with chitosan (Grillo et al., 2014 a, b). The nanoformulation of hexaconazole was found to release a maximum amount of pesticide within 14 days in a non Fickian manner (Chauhan et al., 2017). Sustained release was observed for more than 30 days in nanoformulation of insecticide nicotine hydrochloride (NCT) (Yang et al., 2018). The release profile of paraquat from the nanoparticle polymeric matrix showed sustained release upto 17 days. Application of the Korsmeyer–Peppas mathematical model to the release kinetics data indicated that the release

was governed by anomalous transport (a combination of diffusion and Case II transport) (Grillo et al., 2014a).

4.4.4 Release mechanism of cartap from chitosan alginate nanospheres

When evaluating the nanoparticle stability, effectiveness, and efficiency, the *in vitro* release kinetics of nanospheres is an important parameter to consider as it offers crucial details about their ability to alter their release profile. A controlled release pattern was observed for the nanoformulation and from cumulative release percentage of cartap it was found that the maximum time of release for the nanoformulation was 12 days. More than half of the cartap was released within 5 days from the formulation. Five different mathematical models: zero order, first order Higuchi, Korsmeyer-Peppas and Hixson were employed to study release kinetics of insecticide from encapsulated nanospheres. The results of linearization of the release curves for encapsulated cartap are shown in Fig. 4.55-4.60. Linear regression was used to calculate the values of the release constant and release exponent (Table 4.4).

Table 4.4: Parameter values obtained by fitting of mathematical models to the data for the chitosan alginate nanoparticles containing insecticide cartap hydrochloride

	Release constant (k) min ⁻¹	Release exponent (n)	Correlation coefficient (r)
Zero order	25.18	0.165	0.814
First order	1.894	-0.001	0.952
Hixson	0.401	0.004	0.92
Higuchi	10.68	3.785	0.91
Peppas	0.149	1.235	0.738

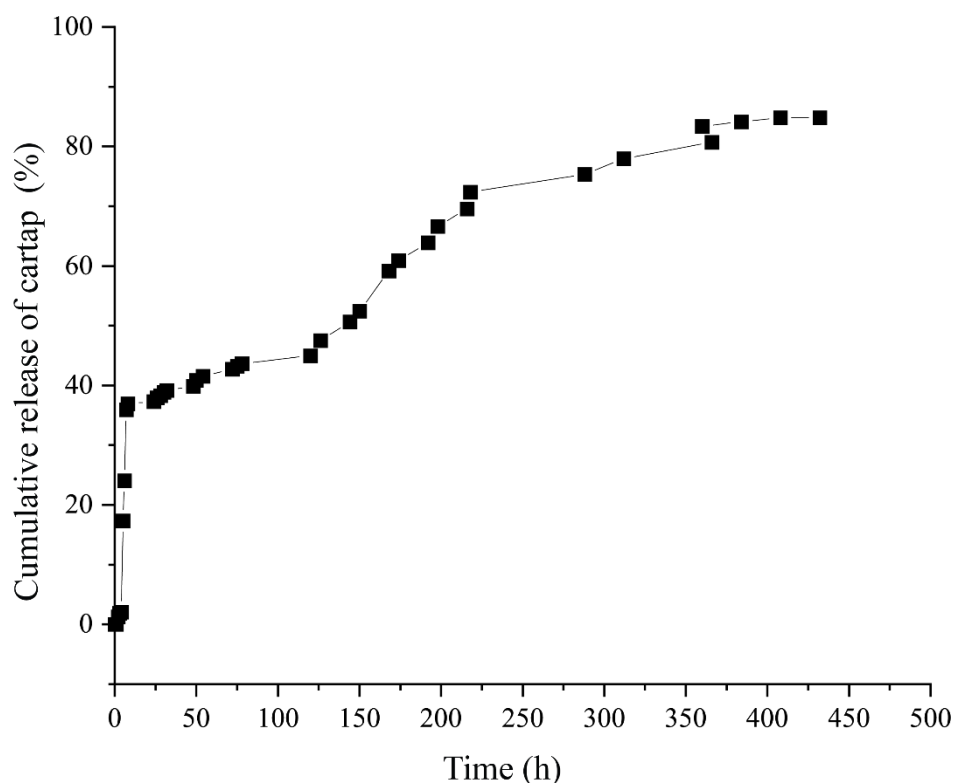


Fig. 4.55: *In vitro* release kinetics profile of cartap loaded nanospheres.

In zero order kinetics, by plotting cumulative insecticide release vs. time and calculating the slope of the curve, it was found that the r^2 value was approaching 0.814 (Fig. 4.56), indicating that cartap release from the matrix did not follow zero-order release kinetics (Agnihotri et al., 2012). In first order release, linear kinetics was followed and cartap release from in-vitro dissolution study was plotted between log of remaining insecticide (%) vs. time and slope of plot gives value of r^2 (0.952) (Fig. 4.57) which confirmed that insecticide release from the matrix followed first order release kinetics (Agnihotri et al., 2012).

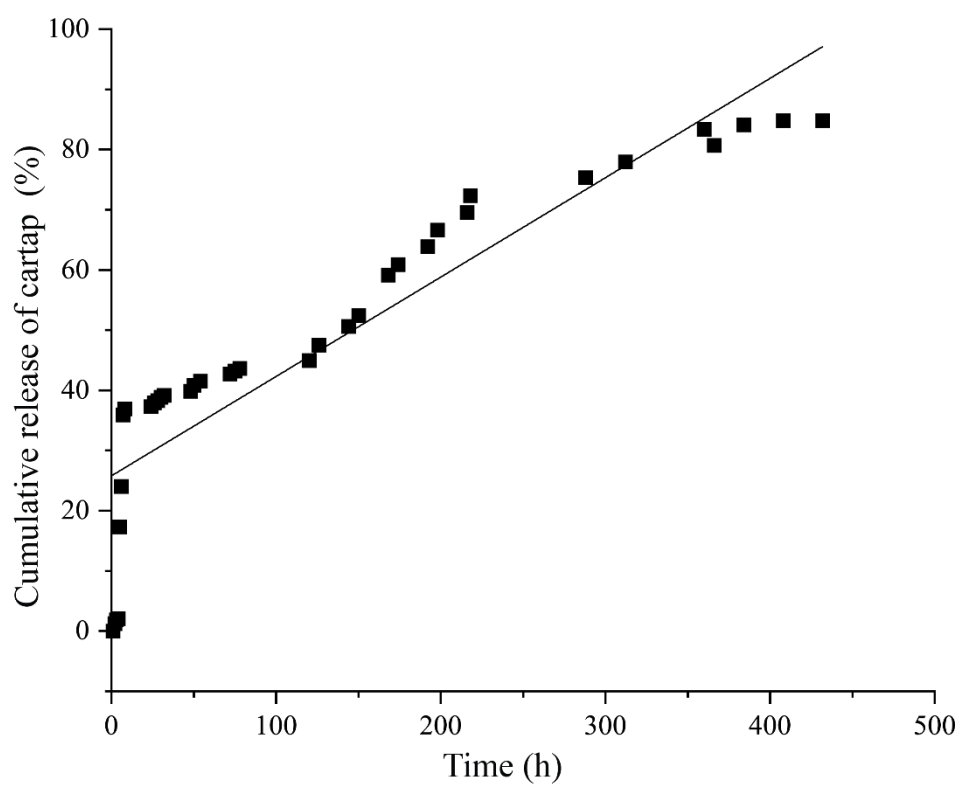


Fig. 4.56: Analysis of cartap release from nanospheres using zero order kinetics

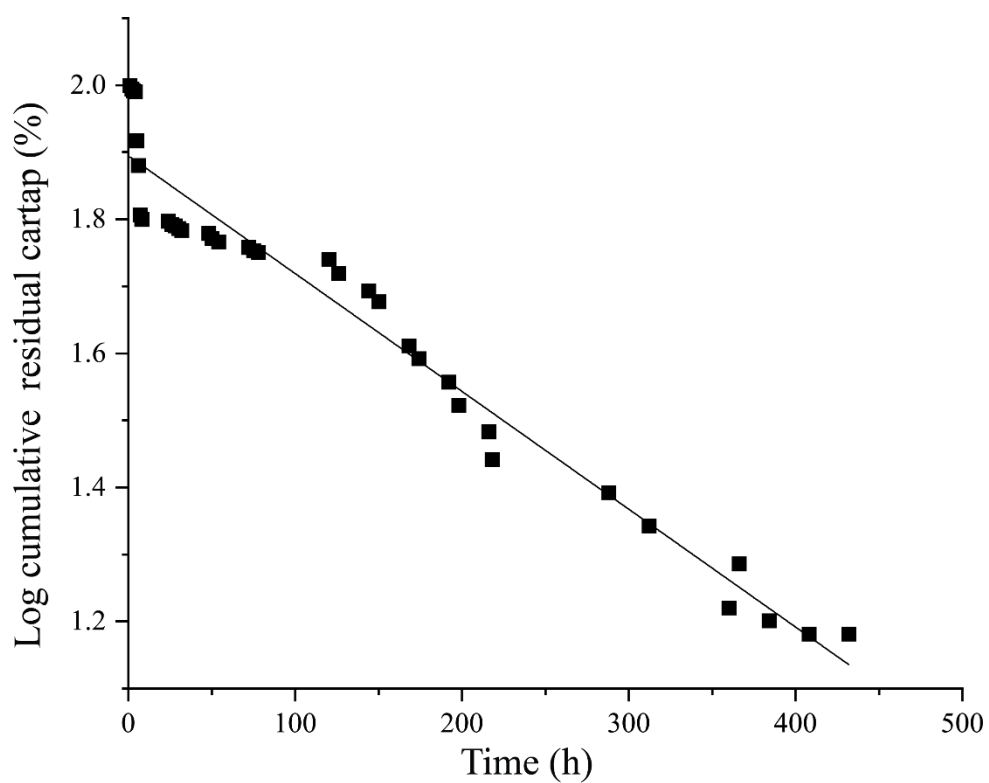


Fig. 4.57: Analysis of cartap release from nanospheres using first order kinetics

The best model to study release kinetics from controlled release systems is Higuchi model which includes both diffusion and dissolution. In this model, the plot of cumulative insecticide release (%) versus square root of time was generated which gives slope of plot (correlation coefficient $r^2 = 0.91$) (Fig. 4.58) indicated that release of from the matrix obeyed Higuchi model (Dash et al., 2010). The Hixson-Crowell cube root law describes the release from system where there is a change in surface area and diameter of particles. To study the release kinetics of insecticide, a graph was plotted between the cube root of the insecticide remaining (%) versus time and from slop of graph the value of correlation coefficient ($r^2 = 0.92$) (Fig. 4.59) revealed that change in diameter and surface area of the nanospheres with the progressive dissolution had little effect on release (Subal., 2006).

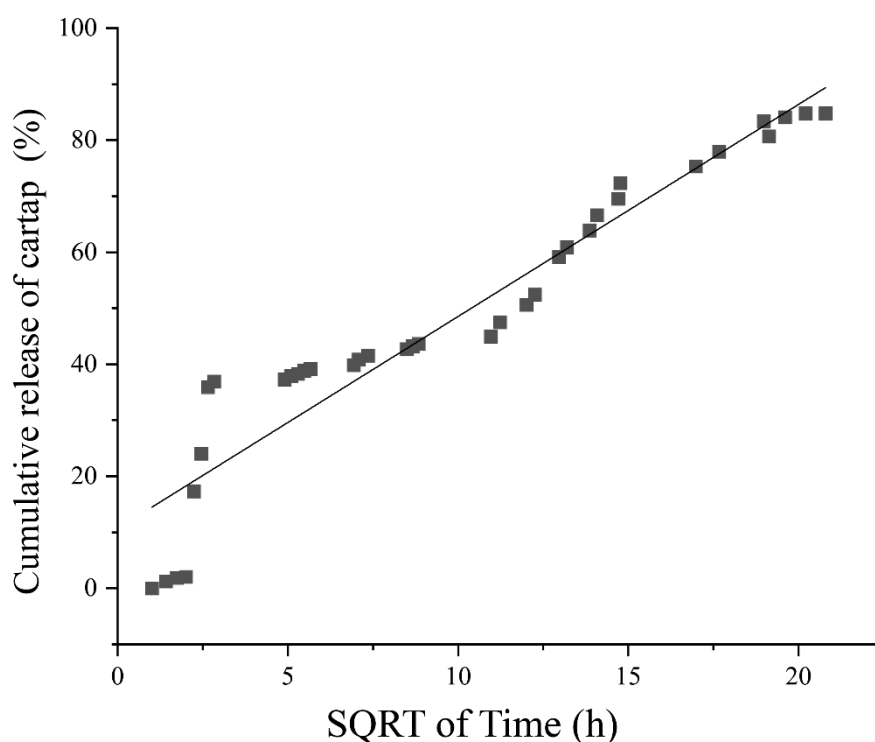


Fig. 4.58: Kinetic analysis of cartap release from nanospheres using Higuchi model

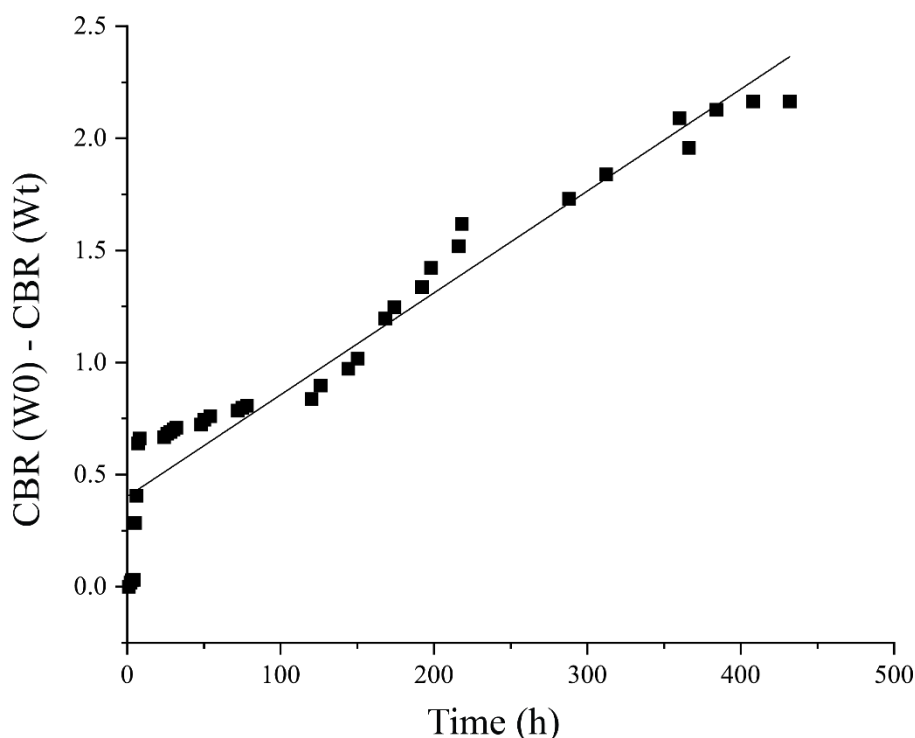


Fig. 4.59: Kinetic analysis of cartap release from nanospheres using Hixson Crowell model

The release of cartap from nanospheres obeyed Higuchi, first order kinetics and Hixson model. The goodness of fit for these models was ranked in order: first-order >Hixson-Crowell >Higuchi. When it's verified that insecticides were released by diffusion, the next step is to figure out the form of diffusion. Korsmeyer and Peppas model was used to understand type of diffusion. The plot for release data was generated between cumulative insecticide release (%) vs. log time and n value calculated from slope was used to identify different release mechanisms. Diffusion constant (n) determines the mechanism of insecticide release from the nanoformulation. Fickian and non-Fickian behavior was defined by n value. Case I transport: $n \leq 0.43$ indicates a Fickian release, implying that diffusion is the primary regulator of the process. Case II transport: $n = 0.85$ denotes polymer relaxation process while super case II transport: $n < 0.85$ was driven by both relaxation and erosion. Intermediate values of n ($0.43 < n < 0.85$) implies non-Fickian release in which both relaxation and diffusion occur simultaneously (Singhvi and Singh, 2011; Ritger and Peppas, 1987).

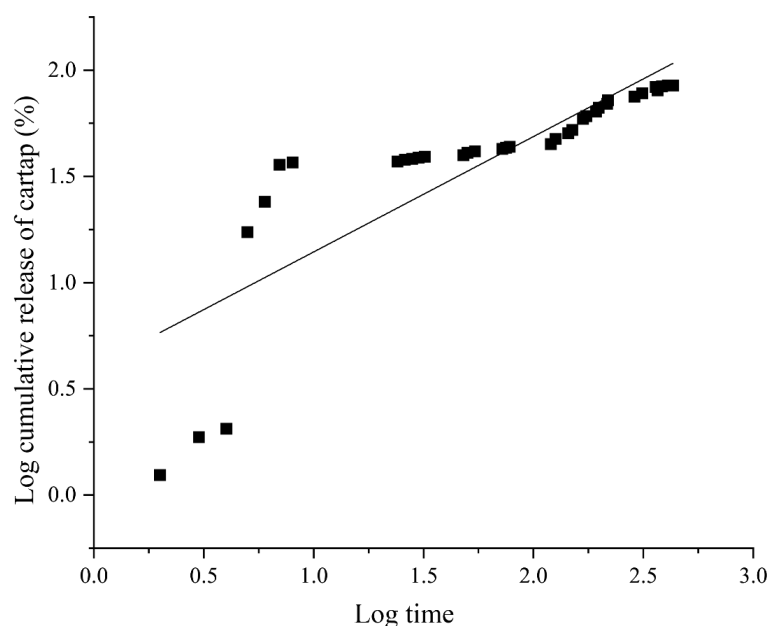


Fig. 4.60: Kinetic analysis of cartap release from nanospheres using Korsmeyer Peppas model

The release coefficient calculated by the Peppas model was 1.23 (Fig. 4.60), indicating that cartap was released via super Case II transport, reflecting a new mode of interaction between the insecticide and the nanospheres. In super case II transport, release was controlled by macromolecular relaxation of polymeric chains of chitosan and by erosion process. The dispersing medium penetrates into the system, forms pore and then erodes it. (Singhvi and Singh, 2011).

Significant differences were seen between the release profiles of encapsulated and unbound paraquat in a comparable investigation (Dos et al., 2010). Maruyama et al demonstrated that imazapic was released via diffusion following Fick's law, but imazapyr was released via Case II transport, indicating a different method of interaction between the imazapyr and the nanospheres (Maruyama et al., 2016). Hexaconazole nanoformulation was released in non Fickian manner within 14 days (Chauhan et al., 2017). Grillo et al reported an alteration in the release profile of paraquat and atrazine when the particles were coated with chitosan (Grillo et al., 2014a, b). The release of acetamiprid followed non Fickian behavior at pH 4, 7 and 10 (Kumar et al., 2015). Paraquat nanospheres followed anomalous transport (a combination of diffusion and Case II transport) and showed sustained release upto 17 days (Janes et al., 2001).

In the present study, cartap hydrochloride induced stress was studied on physiological parameters of *Anabaena variabilis* ARM 441. Initially, to check the tolerance level of cyanobacteria checked by treating it with different concentration ranging from 0-100 $\mu\text{g ml}^{-1}$ cartap. The cyanobacteria tolerated upto 30 $\mu\text{g ml}^{-1}$ cartap and growth was ceased at 40 $\mu\text{g ml}^{-1}$. At this concentration, pigments were released in the medium due to cell lysis resulting in colorless culture. Three concentrations of cartap 5 $\mu\text{g ml}^{-1}$ (half of recommended dose), 10 $\mu\text{g ml}^{-1}$ (recommended dose) and 20 $\mu\text{g ml}^{-1}$ (double of recommended dose) were selected for further experimentation. Growth of the cyanobacteria showed a decline in dose dependent manner. In 5, 10 and 20 $\mu\text{g ml}^{-1}$, the growth was declined to 30.93, 57.31, and 96.99% respectively on day 21. With increase in insecticide dose, specific growth rate decreased while increasing generation time of cyanobacterium. Increased concentrations of cartap resulted in a decrease in total protein content, heterocyst frequency, dry weight biomass and specific growth rate constant of the test microorganism, which appears to be due to interference of cartap with the structure and functions of photosynthetic machinery, resulting in depletion of its energy reserve, which had an adverse effect on its growth and development. The production of photosynthetic pigments was inhibited in same way as growth of *Anabaena variabilis* ARM 441 upon cartap treatment. Chlorophyll *a* at the end of day 7 showed decreasing trend from 37.22 to 98.73% at 5- 20 $\mu\text{g ml}^{-1}$ of cartap, whereas carotenoids decreased by 30.55- 72.22%, respectively. As most of the carotenoid synthesizing enzymes are membrane-bound, reduction of carotenoid biosynthesis might be due to the interaction of the insecticide with these enzymes. The decrease in phycobiliproteins content of the microorganism at higher concentration of cartap may be ascribed directly to the inhibition of pigment synthesis by the cartap or due to accelerated degradation of pigments due to increased level of active oxygen species at various sites of the photosynthetic electron transport chain during insecticide stress. At recommended dose of cartap (10 $\mu\text{g ml}^{-1}$), photosynthetic pigments showed decline in the order of Chl *a* > APC > PC > Car > PE on day 7. The effect of cartap was more pronounced on photosynthesis than respiration of test microorganism. When grown in presence of 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap, total nitrogen content declined by 17.28, 24.29 and 36.44% respectively. This suggests that cyanobacterium is able to contribute some nitrogen through nitrogen fixation even under stress conditions. As cyanobacteria use nitrate, nitrite or ammonium as nitrogen source for their growth and development, the effect of cartap on nitrogen metabolism of *Anabaena variabilis* was studied. The cyanobacterium exhibited decrease in nitrate and

ammonium uptake in presence of cartap however, nitrite uptake was slightly affected. The cartap significantly affected nitrate and nitrite reductase activity. The low level of uptake of nitrate and nitrite by the cyanobacterium in presence of cartap may be the possible reason for low activities of these enzymes. Decrease in GS activity of cyanobacterium can be correlated with reduced nitrite and nitrate uptake rather than direct effect of cartap on activity of glutamine synthetase. The effect of cartap was strongest on NiR activity, followed by GS activity, and NR activity, among these three enzymes. Insecticide toxicity leads to the formation of free radicals and cyanobacteria respond to this stress by activating antioxidant enzymatic defence system (SOD, POD, Catalase and GST). Cartap presence increased the SOD activity by 69.05 - 129.05%; POD activity by 62.5 - 287.5%; catalase activity by 8.33 - 216.66% and GST activity by 33.26 - 233.04 % at 5, 10 and 20 $\mu\text{g ml}^{-1}$ of cartap respectively. The increase in activities of these enzymes of cyanobacterium in presence of cartap suggested the operation of enzymatic defence mechanism by the microorganism to counter oxidative stress caused by cartap. The production of superoxide radical (SOR), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) was stimulated in *Anabaena variabilis* with cartap treatment. Glutathione and ascorbate levels were significantly in a concentration and time dependent manner. The results suggested that cyanobacterium was contouring the cartap stress by activating both enzymatic as well as non-enzymatic pathways. Proline and sucrose content in *Anabaena variabilis* increased in dose dependent manner. To check whether the cyanobacterium was resistant to cartap or utilized it intracellularly, cyanobacterium was grown in medium supplemented with cartap concentration of 5, 10, 30 $\mu\text{g ml}^{-1}$. The cyanobacterium removed cartap by 67, 55.8, and 50.43% in 5, 10, 30 $\mu\text{g ml}^{-1}$ after 24 h.

Since the *Anabaena variabilis* was affected at 10 $\mu\text{g ml}^{-1}$ of cartap which is recommended field application dose so an attempt has been done to synthesize chitosan tripolyphosphate and chitosan alginate nanoparticles loaded with cartap hydrochloride for controlled delivery of insecticide. Chitosan nanospheres (CS-TPP) were synthesized through ionic gelation method using sodium tripolyphosphate as crosslinker. The particles were stable upto 30 days with average size of 201.35 ± 3.65 nm in CS-TPP nps and 263.87 ± 4.58 nm in C-CS-TPP nps. Similarly, the polydispersity values for CS-TPP nps was 0.286 ± 0.002 and in insecticide loaded C-CS-TPP nps it was 0.271 ± 0.004 . The zeta potential of CS-TPP nps was $+40.96 \pm 1.25$ and $+43.6 \pm 0.260$ mV in C-CS-TPP nps. About 86.05% of entrapment efficiency was found for cartap loaded nanospheres. FTIR, XRD and FESEM confirmed the encapsulation of insecticide into the nanospheres. Similarly, Alginate–chitosan nanospheres were prepared

in a two-step procedure based on the ionotropic gelation of polyanion with calcium chloride followed by polycationic crosslinking. Size distributions of the synthesized nanospheres were evaluated by dynamic light scattering. The average size of the blank nanospheres (CS-ALG) using the DLS was 214.6 ± 4.477 nm but after incorporation of cartap, the size of loaded (C-CS-ALG) nps was increased to 381.77 ± 0.183 nm. Similarly, the polydispersity values for CS-ALG nps was 0.218 ± 0.004 and for insecticide loaded C-CS-ALG nps, it was 0.356 ± 0.013 . The zeta potential of CS-ALG nps was $+22.66 \pm 1.039$. However, cartap incorporation in the nanospheres resulted in increased stability, with higher zeta potential $+31.2 \pm 1.327$ mV in C-CS-ALG nps. Cartap loaded chitosan nanospheres had an entrapment efficiency of 76.19%. FTIR, XRD and FESEM confirmed the encapsulation of insecticide into the nanospheres.

The *in vitro* release kinetics of nanospheres offers crucial details about their ability to alter their release profile, and is therefore an important parameter to consider when evaluating the nanoparticle stability, effectiveness, and efficiency. The cumulative release percentage of cartap from C-CS-TPP nanoformulations was calculated and found that the maximum time of release for the nanoformulation was 15 days. Five different mathematical models were employed to study release kinetics of insecticide from encapsulated nanospheres. The goodness of fit for these models ranked in order: first-order > Higuchi > Hixson-Crowell. Release of cartap from nanospheres was by means of non-Fickian diffusion. In case of C-CS-ALG nanospheres, a controlled release pattern was observed for the nanoformulation and from cumulative release percentage of cartap it was found that the maximum time of release for the nanoformulation was 12 days. The goodness of fit for these models was ranked in order: first-order > Hixson-Crowell > Higuchi. The release coefficient calculated by Peppas model was 1.23, indicating that cartap was released via super Case II transport, reflecting a new mode of interaction between the insecticide and the nanospheres. Comparative analysis of these synthesized nanospheres showed that chitosan tripolyphosphate nanoparticles were more superior to chitosan alginate nanospheres in terms of their release of cartap in the environment. But both types of nanoformulations because of their biodegradability, biocompatibility, greater stability, low toxicity and simple synthesis process, offers valuable tool for pesticide delivery systems as use of reduced quantity of insecticides, as well as their less frequent applications in crop cultivation is one of the primary objectives of sustainable agriculture to minimize toxicity to non-target organisms and associated risk of wider environmental contamination.

1. The cyanobacterium survived up to 30 $\mu\text{g ml}^{-1}$ cartap and did not grow above 40 $\mu\text{g ml}^{-1}$ cartap. Concentration-dependent decrease in growth was observed in presence of cartap.
2. The production of photosynthetic pigments was inhibited at recommended dose of cartap (10 $\mu\text{g ml}^{-1}$), in the order of Chl *a* > APC > PC > Car > PE.
3. Cartap inhibited oxygen consumption (respiration) of *Anabaena variabilis* in same way as photosynthesis.
4. Total nitrogen content of cyanobacterium was inhibited in dose dependent manner which implies that cyanobacterium is able to contribute some nitrogen through nitrogen fixation even under stress conditions.
5. The effect of cartap was strongest on Nitrite reductase activity, followed by Glutamine synthetase activity, and Nitrate reductase activity, among these three enzymes.
6. Antioxidant defense system of cyanobacterium was activated due to cartap stress. Elevated levels of POD, SOD, CAT and GST activity in the ARM 441 strain indicates that these enzymes are helping the organism to cope with cartap stress.
7. The amount of SOR and H_2O_2 production was comparatively higher in 20 $\mu\text{g ml}^{-1}$ cartap than 5 and 10 $\mu\text{g ml}^{-1}$ of insecticide.
8. Osmolyte production and Lipid peroxidation were also increased due to cartap stress.
9. The cyanobacterium removed 67, 55.8, and 50.43% of cartap in in 5, 10, 30 $\mu\text{g ml}^{-1}$ of cartap supplemented medium after 24 h of incubation.
10. SEM analysis revealed that external morphology of *Anabaena variabilis* was disrupted and damaged, when treated with different doses of cartap.
11. Chitosan tripolyphosphate nanospheres loaded with cartap were synthesized through ionic gelation method showed encapsulation efficiency of 86.05% and release cartap from nanoformulation by means of non-Fickian diffusion mechanism.
12. Alginate–chitosan nanospheres incorporated with cartap were by ionotropic gelation followed by polycationic crosslinking had an entrapment efficiency of 76.19% and release of cartap from insecticidal nanoformulation followed super Case II transport mechanism.
13. Chitosan tripolyphosphate nanoparticles were more superior to chitosan alginate nanospheres in terms of their release of cartap in the environment.
14. Both types of nanoformulations offers valuable tool for pesticide delivery systems as use of reduced quantity of insecticides and are biodegradable.

Only 0.1% of the applied agrochemicals are delivered to the particular target site and act effectively against the target organism and rest posing a threat to living organisms in the soil and rivers due to losses during their application via degradation, volatilization, photolysis and leaching. Indiscriminate application of pesticides has resulted in its persistence in the environment which has impacted non-target microorganisms. Nanoformulation of cartap hydrochloride has been shown to have potential to solve this problem of pesticide contamination of environment as these are controlled release formulations and are biodegradable, biocompatible, greater stability, low toxicity. These qualities made them valuable tool for pesticide delivery systems as use of reduced quantity of insecticides, as well as their less frequent applications in crop cultivation is one of the primary objectives of sustainable agriculture to minimize toxicity to non-target organisms and associated risk of wider environmental contamination.

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Publications (accepted and published)

1. **Kaur, I.**, Agnihotri S. & Goyal, D. (2021). Formulation of cartap hydrochloride crosslinked chitosan tripolyphosphate nanospheres and its characterization. *Colloid and polymer science*. (SCI, IF-1.931)
2. **Kaur, I.**, Agnihotri S. & Goyal, D. (2021). Fabrication of chitosan-alginate nanospheres for controlled release of cartap hydrochloride. *IOP Nanotechnology*. (SCI, IF-3.874)
3. **Kaur, I.**, & Goyal, D. (2021). Cartap hydrochloride induced stress response in *Anabaena variabilis* ARM 441. *Pesticide Biochemistry and Physiology*, 104904. (SCI, IF-3.963)

Conferences attended

1. 4th International Conference on Recent Trends in Bioengineering (ICRTB-2021) 12-13 Feb, 2021.

Composition of BG-11 medium (Stanier *et.al*, 1971)

Constituent's	Quantity (g/l)
Sodium nitrate	1.5
K ₂ HPO ₄	0.040
MgSO ₄ .7H ₂ O	0.075
CaCl ₂ .H ₂ O	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA	0.001
Sodium carbonate	0.020
Trace metal mix	1 ml
Distilled water	1000 ml

Trace metal mix composition

Constituents	Quantity (g/l)
H ₃ BO ₃	2.86
MnCl ₂ .4H ₂ O	1.81
ZnSO ₄ .7H ₂ O	0.222
Na ₂ MoO ₄ .2H ₂ O	0.39
CuSO ₄ .5H ₂ O	0.079
Co(NO ₃) ₂ .6H ₂ O	0.0494
Distilled water	1000 ml

Protein estimation

1. Solution A: 2% sodium carbonate in 0.1 N NaOH
2. Solution B: 0.5% copper sulphate in 1% sodium potassium tartrate
3. Solution C: Obtained by mixing 50 ml solution A and 1 ml of solution B (prepared fresh)
4. Solution D: Folin's reagent (1:1 diluted commercial Folin's reagent) prepared fresh

Nitrate estimation

Brucine: 5 gm in 100 ml of glacial acid.

Nitrite estimation

1. Sulphanilamide (1%): 1 gm solid sulphanilamide was dissolved in acid solution made up with 75 mL of distilled water and 25 ml concentrated hydrochloric acid.
2. N-(1-Naphthyl) ethylenediamine hydrochloride (0.02% w/v)

Both reagents were stored in amber coloured reagent bottles.

Ammonium estimation

1. Phenol- alcohol solution: gm phenol was dissolved in 100 ml of 95% ethanol.
2. Sodium nitroprusside solution: 1 gm of sodium nitroprusside was dissolved in 200 ml of distilled water.
3. Alkaline solution: 100gm of tri-sodium citrate and 5 gm of sodium hydroxide were dissolved in 500 ml of distilled water.

Oxidizing solution: This solution was prepared fresh at the time of conducting experiment.

Alkaline solution (100 ml) and sodium hypochlorite (25 ml) were mixed together.

Nitrate reductase (NR) and Nitrite reductase (NiR) activities

1. Sodium dithionite: 0.1 M dissolved in 0.3M NaHCO₃
2. Sulphanilamide (1%): 1 gm solid sulphanilamide was dissolved in acid solution made up with 75 ml of distilled water and 25 ml concentrated hydrochloric acid.

Glutamine synthetase (GS) activity

1. Stop mixture : this was prepared by adding 4 mL ferric chloride (10%), 1 ml of trichloro- acetic acid (24%), 0.5 ml of 6 N HCl and 6.5 ml of double distilled water.
2. Assay mixture: A two folds assay mixture was prepared by adding 2 ml of imidazole buffer, 15 ml of glutamine, 1.5 ml of manganese chloride, 1.0 ml of ADP, 1 ml of sodium arsenate and 1.5 ml hydroxylamine hydrochloride. Hydroxylamine hydrochloride was neutralized before adding to other components with 1.5 ml of 2 N sodium hydroxide.

Malondialdehyde (MDA) content

1. Thiobarbituric acid (TBA), 0.5% prepared in 20% TCA

Glutathione (GSH) content

1. DTNB (2, 2'-Dinitro-5 – dithiodibenzoic acid) 5 mg in 25 mL of 0.1% sodium citrate solution.

Ascorbate (ASA) content

1. Dye(2,6- Dichloroindophenol): Dissolved 0.014 gm dye in 500 ml H₂O and stored in dark at 4°C

Proline content

Acid ninhydrin: Dissolved 1.25 g ninhydrin in 30 ml glacial acetic acid and added continuously 20 ml of 6 M phosphoric acid with agitation. Stored at 4 °C.

Cartap estimation

1. DTNB (2, 2'-Dinitro-5, 5' - dithiodibenzoic acid): 50 mg in 100 ml of methanol.
2. Buffer solution:
Phosphoric acid, 0.5 M
Boric acid, 0.5 M
Acetic acid, 0.5 M
Add an equal volume of each solution and adjust the pH to 9.0 with 0.2 N sodium hydroxide solution. Diluted it 4 times with double distilled water and made 100 ml final volume.